A BIOCHEMICAL STUDY OF NITROGEN IN CERTAIN LEGUMES

BY

ALBERT LEMUEL WHITING
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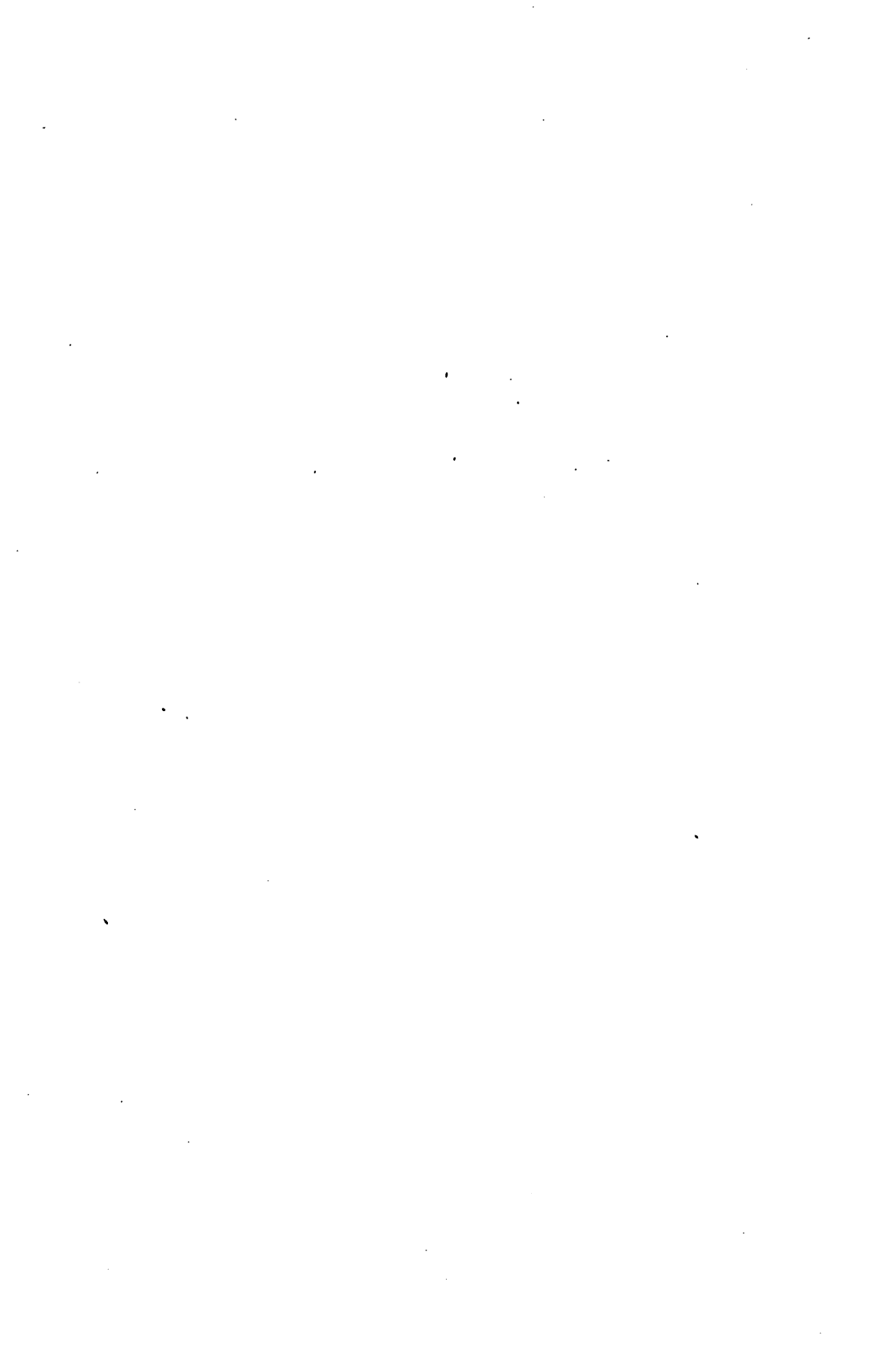
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By ALBERT L. WHITING

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ILLUSTRATIONS

A BIOCHEMICAL STUDY OF NITROGEN IN CERTAIN LEGUMES[1]

By ALBERT L. WHITING, ASSOCIATE IN SOIL BIOLOGY

INTRODUCTION

The investigations considered in this publication bear on the biochemical nature of the element nitrogen, especially as concerns its fixation and assimilation thru the symbiotic relationship of *Bacillus radicicola* and certain members of the botanical family known as Leguminosae.

The sources of the element nitrogen available for agricultural purposes are numerous. Of these the atmosphere is by far the most important and most extensive. Above each acre of the earth's surface there are about 69 million pounds of atmospheric nitrogen, and science has shown that by thoroly scientific systems of management this nitrogen may be appropriated for soil improvement at a minimum expense. By growing legumes, atmospheric nitrogen may be obtained at a low cost, often at no net cost, for most agricultural leguminous crops are worth growing for feed or seed alone. In commercial fertilizing materials, nitrogen costs from fifteen to twenty cents per pound, an amount from two to five times greater than that expended for any of the other essential elements of plant food. It is of passing interest to note how greatly disproportionate the cost values of these elements are to the relative supplies, when the nitrogen in the air is considered.

The United States spends annually, abroad, over 32 million dollars in the purchase of combined nitrogen for use in various operations, agricultural and otherwise.[2] Of this amount 16½ million dollars are expended for the purchase of sodium nitrate, which is the

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[1]Submitted to the Faculty of the Graduate School of the University of Illinois in partial fulfilment of the requirements for the degree of doctor of philosophy, June, 1912. Revised to date of issuance.

[2]Norton: Special Agent Series, Dept. of Commerce and Labor, Bur. of Manfr., No. 52, 9-11.

most important commercial form of inorganic nitrogen. The present world supply of this salt is estimated at 454,576,200,000 pounds.[1]

How insufficient this supply is, when measured by crop requirements, may be realized from the fact that the following nine important crops of the United States,—corn, wheat, oats, barley, rye, potatoes, hay, cotton, and tobacco, in the year 1910, required for their growth 11,500,000,000 pounds of nitrogen.[2] If sodium nitrate were used for growing these crops at the rate stated above, the supply would be exhausted in about six years. On the other hand, the nitrogen above only one square mile, weighing 20 million tons, would be sufficient to supply what the entire world, at its present rate of consumption, would require for the next fifty years.[3] The nitrogen above four acres would furnish more than the actual yearly consumption of commercial nitrogen in the entire United States.

The wonderful possibilities presented by such an extensive source of plant food, and the fact that over 100 million dollars are invested in commercial fertilizers each year in the United States, a large part of which is wasted or uselessly applied, together with the great natural losses of nitrogen that occur, tend to emphasize greatly the need of a proper utilization of this unlimited reserve supply. Further, it is well recognized that the maintenance of the nitrogen supply is the greatest of our soil problems. Nitrogen cannot be purchased on the market at a price that will permit its extensive application in growing the important crops of the United States. There is only one logical and inexhaustible source of nitrogen for the world to utilize in the production of crops. That source is the atmosphere, from which nitrogen is most economically and easily secured as a result of the symbiotic relationship between *B. radicicola* and leguminous plants.

HISTORICAL

For several centuries certain plants of the Leguminosae have been used as soil improvers. A few of the more important references to their uses are considered here.

In Roman literature, among the works of Columella,[4] mention is made of the Roman farmers regarding beans as possessing the property of enriching the soil, and attention is also called to the practice of plowing under lupines. Alfalfa and vetches were observed to produce similar results to those of lupines and beans. Like notations

[1]Review of Reviews, April, 1910.

[2]Yields taken from U. S. Yearbook, 1910. For calculations see Hopkins' "Soil Fertility and Permanent Agriculture" (1910), 154; also 603-604.

[3]Norton: Special Agent Series, Dept. of Commerce and Labor, Bur. of Manfr., No. 52, 9-11.

[4]Marshall: Microbiology (1911), 273.

may be found among the writings of Thaer and Walz.[1]   Gasparin[2] constantly calls attention to the power of leguminous plants to add nitrogen to the soil.   Jethro Tull[3] wrote concerning the efficiency of legumes in restoring depleted soils, mentioning especially sanfoin and alfalfa.

It may be noted here that Hellriegel, who later was most prominent in the discovery of the relation existing between legumes and bacteria, wrote in 1863 as follows: "Clover plants may develop normally and completely in mere sand to which the necessary mineral constituents of plant food have been added in assimilable forms, even when this soil contains no trace of any compound of nitrogen or of organic matter."

Schultz-Lupitz[4] in 1881 reported results that were of both chemical and practical significance.   After growing lupines for fifteen consecutive times on a sandy soil, without the application of nitrogenous materials, he observed that the yields did not diminish; and when he grew cereals on the same land after the lupines, he found that the yields of the grains were two and three times the yields where no lupines had been grown.   Analyses of the soils at the end of this time showed that where the lupines had been grown, the nitrogen content of the surface six inches had increased by .06 percent.   Frank[5] verified these results with twenty years of lupine culture on the same fields.

About this time a great deal of interest centered on pot-culture experiments with legumes.   Many physiologists and chemists worked on the problem of nitrogen collection by legumes.   Prominent among these were the scientists Boussingault,[6] and Lawes, Gilbert, and Pugh,[7] who, owing to their great accuracy, sacrificed the possibility of becoming the discoverers of this important relationship.   In their great care, they destroyed the vital agency (*B. radicicola*) necessary for the accomplishment of this symbiotic fixation.

Later, in 1886, Hellriegel and his co-worker Wilfarth[8] made the classical discovery that legumes obtain atmospheric nitrogen thru the association of microörganisms living in the nodules.   In a preliminary report read before a section of scientists assembled on September 20, 1886, at Berlin, Hellriegel announced his findings; and in a more com-

[1]Storer: Agriculture (1906), 2, 97.

[2]Ibid.

[3]Lipman: Bacteria in Relation to Country Life (1908), 206.

[4]Schultz-Lupitz: Landw. Jahrb. (1881), 10, 777.

[5]Frank: Landw. Jahrb. (1888), 17, 501.

[6]Boussingault: Ann. Sci. Agron. (1909), 26, Ser. 3, 4, 102-130.

[7]Lawes, Gilbert, and Pugh: Rothamsted Experiments (1905), 6-7.

[8]Hellriegel and Wilfarth: Tagblatt d. Naturforscher Versamml. z. Berlin (1886), 290.

plete account rendered two years later, he made known to the world his researches. These are summarized as follows:[1]

1. The legumes differ fundamentally from the grains in their nutrition with respect to nitrogen.

2. The grains (*Graminteae*) can satisfy their nitrogen need only by means of assimilable combinations existing in the soil, and their development is always in direct proportion to the provision of nitrogen which the soil places at their disposal.

3. Outside the nitrogen of the soil, the legumes have at their service a second source from which they can draw in most abundant manner all the nitrogen which their nutrition demands to complete that lack when the first source is insufficient.

4. That second source is the free nitrogen—the elementary nitrogen of the atmosphere which is furnished to them.

5. The legumes do not possess by themselves the faculty of assimilating the free nitrogen from the air; it is absolutely necessary that the vital action of microörganisms of the soil come to their aid in order to attain this result.

6. In order that the nitrogen of the air can be made to serve the nutrition of the legumes, the sole presence of lower organisms in the soil is not sufficient; it is still necessary that certain among them enter into a symbiotic relationship with the plants.

7. The nodules[2] of the roots must not be considered as simple reservoirs of albuminoid substances; their relation to the assimilation of free nitrogen is that of cause to effect.

Schloesing and Laurent[3] after growing legumes in a confined atmosphere, gave out the following direct evidence of the fixation of atmospheric nitrogen.

```
Atmospheric nitrogen introduced
  into culture vessel.......................2681.2 ccm.
Atmospheric nitrogen withdrawn..............2653.1 ccm.
                                           _____
Amount of nitrogen assimilated..............  28.1 ccm.
                                           (=36.5 mg.)
Nitrogen in the soil and crop................  73.2 mg.
Nitrogen in the soil and seed................  32.6 mg.
                                           _____
Nitrogen assimilated ........................  40.6 mg.
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[1]Hellriegel and Wilfarth: Beil. Zert. d. Verins. für die Rubenzucker industrie, Berlin, Nov., 1888; or Lafar Handbuch der technischen Mykologie (1904-06), 3, 31.

[2]*Nodules* substituted for *tubercles.*

[3]Schloesing and Laurent: Compt. Rend. Acad. Sci. (1890), 111, 750; (1892) 115, 659, 732.

In addition to the scientists mentioned above, Atwater and Woods, Berthelot, Müntz, Ville, Mazé, Dehérain, Frank, Hartig, Nobbe, Hiltner, Warrington, Hopkins, and many others have done much careful work in solving the problem and applying the truths discovered.

BIOLOGICAL

Nodules,[1] which are the visible manifestations of infection, were observed upon the roots of legumes by Malphighi[2] as early as 1687. The investigators of those times believed that the nodules were the result of pathological processes,—that they were lumps, knobs, warts, and even galls. In 1853 the modern conception of the nodule as a normal growth on the legume plant was established by L. C. Treviranus.[3]

Various theories have been proposed as to the function of these peculiar outgrowths, some advancing the idea that they were storage reservoirs or stimuli whereby the plants obtained nitrogen from the atmosphere thru their leaves. Recently Jost[4] has called them "bacterium galls," local hypertrophies not dissimilar to those sometimes caused by animal life. An astonishing conception has crept into the minds of the authors of certain general textbooks on bacteriology and plant physiology that nodules are abnormal growths and that their relationship to plants is either wholly or partially parasitic. It seems preferable, even to those familiar with the limitations of the theory, to describe this relationship as a normal condition and a true mutual symbiosis.

That the formation of these nodules is due to external infection was definitely shown in 1887 by Marshall Ward,[5] who was able to inoculate the roots of young legumes by placing them in contact with old nodules. In Germany the first attempts to grow soybeans (*Glycine hispida*) in the botanical gardens resulted in failure, and it was not until soil from the natural habitat of that plant was imported for inoculation that soybeans were grown successfully.[6] The history of the introduction of alfalfa culture in the states of Kansas and Illinois exemplifies in a large way this need of inoculation. From this experience developed *the soil-transfer method and the glue method*[7] *of inoculation, both of which are recognized today as superior to the use of so-called commercial cultures.*

[1]Nodules are recognized on the following non-leguminous plants: alders (*Alnus glutinosa*), silverberry (*Eleagnus*), sweet gale (*Myrica Gale*), sago palm, an evergreen, (*Podocarpineae*), cycads (*Cycacadeae*), birthwort (*Aristolociaceae*). Nitrogen-fixing bacteria resembling *B. radicicola* have been found in the alder, silverberry, sweet gale, and five varieties of podocarpus.

[2]Malphighi: Op. (1687), 2, 126, Leiden.

[3]Treviranus: Bot. Ztg. (1853), 11, 393.

[4]Jost: Plant Physiology (Gibson 1907), 237.

[5]Ward: Phil. Trans. Roy. Soc. London (1887), 178, 139.

[6]Soil inoculation experiments were instituted as early as 1887 at the Moor Culture Experiment Station, Bremen, Germany.

[7]Ill. Agr. Exp. Sta. Buls. 76, 94.

Two types of nodules have been recognized by Tschirch;[1] Lupinus (lupine) represents one type and Robina (locust) the other. As may be seen by reference to Plates I and II, they differ in morphological appearance. The Lupinus type involves a swelling of the central root cylinders themselves, while in the Robina type only the epidermal and the endodermal tissues seem to enlarge. According to Tschirch, the nodules of lupines alone are of the first type, while those of all other legumes belong to the second.

The figures in Plate III are sufficient to illustrate the most common shapes of the Robina type. The shape varies with the different species of legumes, and to a certain extent with the individuals on the same legume plant. In the experimental work reported in this publication, over twenty thousand nodules were examined closely, and it was not uncommon to find on the same plant notable variations due to external obstructions to growth.

INFECTION

The artificial inoculation of a plant is easily accomplished by contact. If the epidermis of the root is wounded and the infecting organism (*B. radicicola*) brought into contact with the wound, nodules
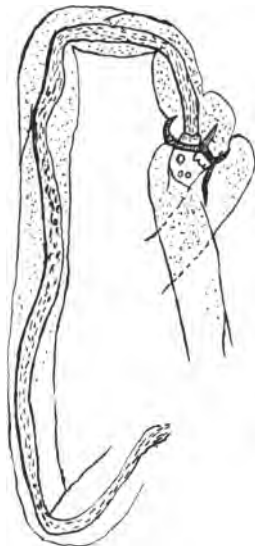
Fig. 1.—Root hair of common pea (*Pisum sativum*), showing infecting strand (x300) (After Prazmowski)
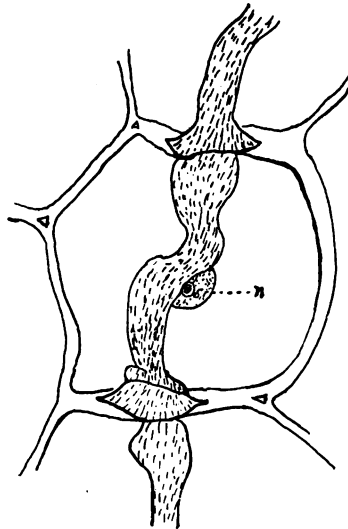
Fig. 2.—Root cell, showing infecting strand passing thru it and the formation of lamellae (x650) (After Prazmowski)

[1]Tschirch: Ber. deut. Bot. Gesell. (1887), **5**, 58.

PLATE I.—NODULES OF ROBINA TYPE ON ROOTS OF SOYBEANS
(Enlarged)

PLATE II.—NODULES OF LUPINUS TYPE ON ROOTS OF LUPINE SEEDLINGS
(After A. Meyer)
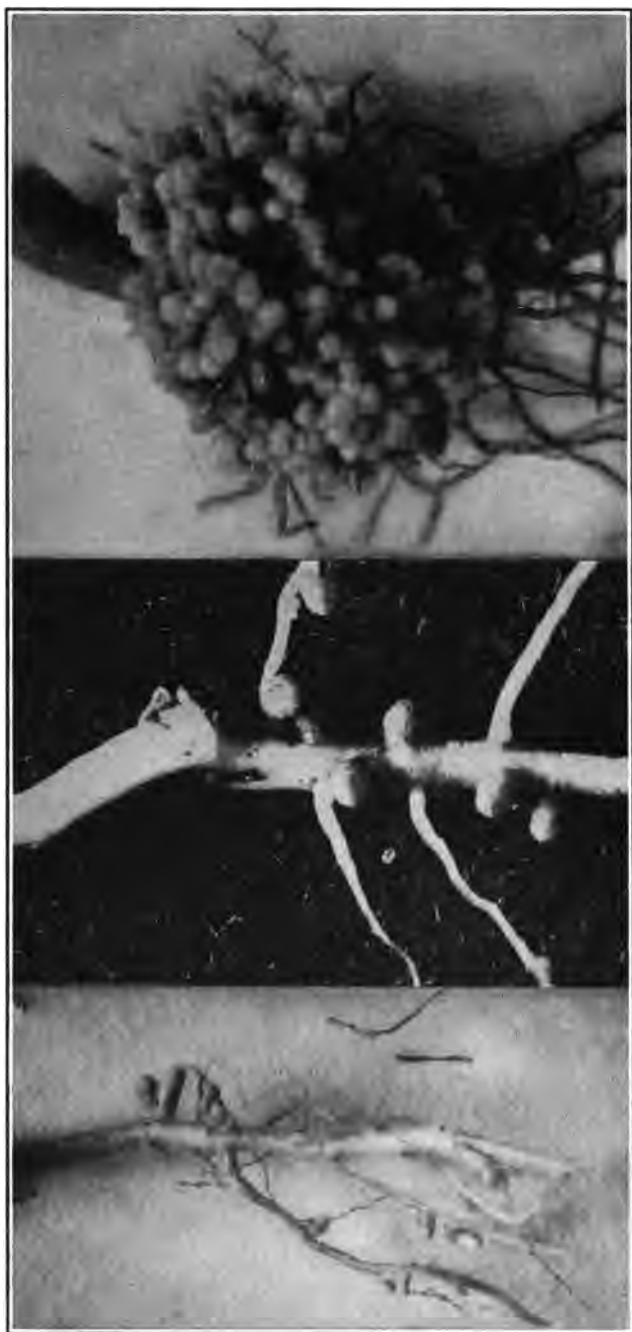
Plate III.—Nodules of Robina Type on (A) Red Clover (*Trifolium pratense*); (B) Vetch (*Vicia sativa*); (C) Sweet Clover (*Melilotus alba*)

result. Inoculation in pot cultures is attained by placing an infusion on the seed or in the medium. A similar method is successful with water cultures.

INOCULATION AS IT OCCURS UNDER FIELD CONDITIONS

Studies of inoculation as it occurs in the field show the following generally accepted phenomena:

As the tip of the root hair of the legume pushes itself out into the soil, it chances to come into intimate contact with the organism *B. radicicola.* Some scientists have exploited the view that the organism is attracted to the plant by chemotaxis, believing that the plant excretes a substance, probably a carbohydrate, which diffuses into the soil solution and attracts the motile organism. While it has been rather definitely shown that this organism progresses in the soil at a rapid rate, nevertheless the number of root hairs infected[1] is too small to lend support to a chemotactic theory. However the case may be, the organisms cluster at the tip of the hair and by means of an enzyme (or otherwise) rapidly dissolve the cellulose of the cell wall, thus enabling the organism to enter the root hair. As a result, there is a decided bending of the tip, causing it to resemble a shepherd's crook. This was early observed as a sign of complete infection. It is claimed that other root hairs which form after infection are immune to the attack of other leguminous bacteria.[2]
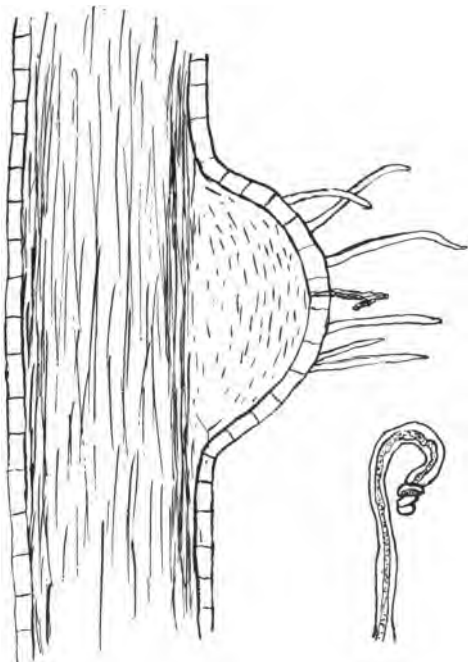
Fig. 3.—Young nodule magnified, showing affected root hair and same root hair more highly magnified (After Atkinson)

The organisms, by rapid division and growth, advance thru the center of the infected root hair. Prazmowski[3] found organisms in the cell

[1]Pierce, G. J.: Proc. Cal. Acad. Sci. II, No. 10 (1902), 295-328. Pierce found the proportion with bur clover to be 1:1000.

[2]Fred: Vir. Agr. Exp. Sta. Ann. Rpt. 1909-10, 123-125.

[3]Prazmowski: Landw. Vers. Stat. (1890), 37, 160-238.

sap and even in the epidermis only two days after inoculation. In this advance an infection strand (Infektion-schlauche) is formed, which consists of gelatinous material, and in the earlier stages of development this strand may be traced from the root hair into the inner tissue of the root and from cell to cell thruout the nodule. This infecting strand is not supposed to constitute a portion of the living tissue, nor is it a well-defined tube; but, as Fred has recently shown, it consists of a large number of zoogloea occurring adjacent to one another, in which separate bacteria can be distinguished. The infecting strand branches profusely, and it was this habit of growth which caused the early investigators to consider it the mycelium of a fungous growth.

Growth of the Nodule

The presence of *B. radicicola* in the tissues of the root causes a rapid cell division in the pericycle. These cells become larger and contain more protoplasm than the surrounding cells, and as growth takes place, the cortical parenchyma and epidermis are forced outward, thus forming a nodule. The growth of the nodule is apical. The various tissues common to the plant are present (see Fig. 4). In the central portion of the nodule is the so-called bacteroidal tissue, which is ochre, flesh, or gray in color, according to the age of the nodule, and in this portion the infecting strand (Infektion-schlauche) is distinguished in the young nodule. It ramifies thruout the cells, causing those which it enters to lose their power of cell division but not of growth. Later, or in older nodules, the infecting strand is not visible, and the bacteroidal tissue loses its firmness. At the period when seed formation is at its height, most of the nodules are soft, and the internal tissues slough off, leaving the more resistant epidermal tissue as a mere shell, which later decays. The endurance of the nodule depends upon several factors,—chiefly, however, upon the kind of legume plant on which it is produced and the need of nitrogen by that plant.

Pierce[1] considers the nodules as originating endogenously from the same layer of cells as the lateral roots, and as being morphologically similar to them; however, as the lateral roots rupture the epidermis, the above statement is not entirely in accord with what actually takes place.

The nodules are largest and most numerous where aeration is best in the soil. In saturated soils they occur at the surface and are often found colored green, very similar to sunburned potatoes. Nodules form in solutions, and exceptionally well in certain nutrient solutions. Several interesting instances have been brought to the attention of the Experiment Station, in which the observers believed that the nodules

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[1]Pierce, G. J.: Proc. Cal. Acad. Sci. II, No. 10 (1902), 295-328.

had grown above the ground. These peculiarities were undoubtedly
caused by unobserved physical conditions occurring at the time of in-
fection or afterward.
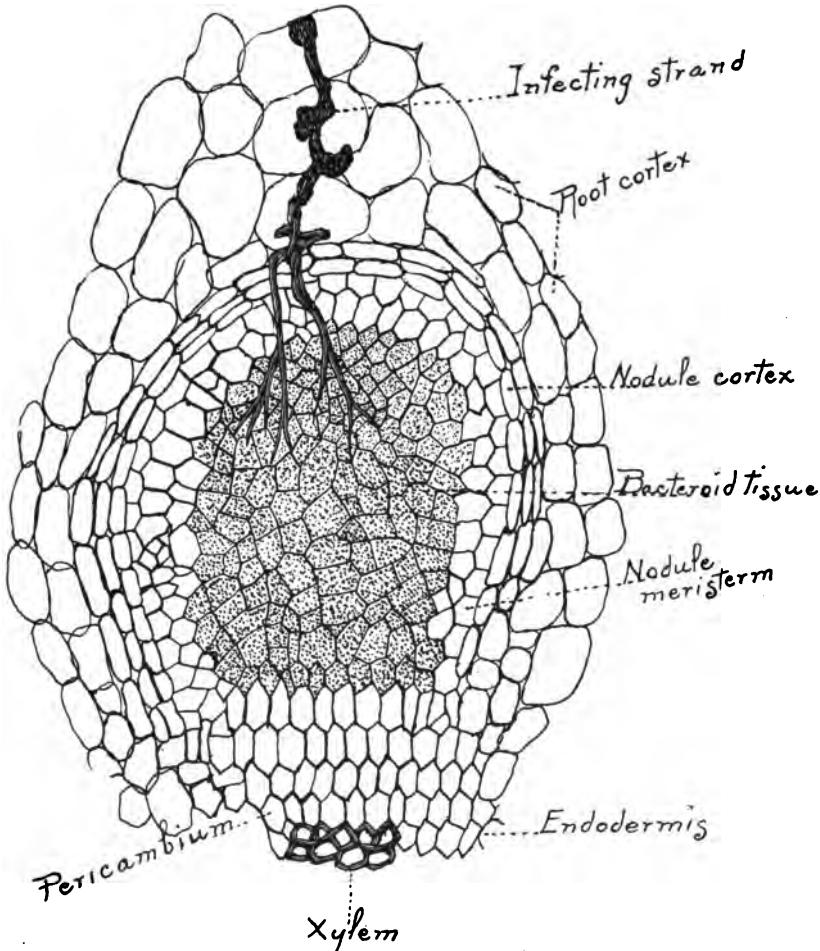
FIG. 4.—YOUNG NODULE, SHOWING THE BEGINNING OF THE DIFFERENTIATION
OF ITS TISSUES (After Prazmowski)

BACTERIOLOGICAL

Minute bodies were first detected in nodules in 1866 by Woronin,[1]
a Russian botanist. At that time bacteria were not recognized, and it
was not until 1887 that they were demonstrated to be true bacteria by
Wigand.[2]

[1]Woronin: Bot. Ztg. (1866), **24**, 329.
[2]Wigand: Bot. Heft. Forsch, a.d.Bot. Bart. 3 Marburg (1887), 288.

Immediately afterward, Beyerinck[1] isolated the organism on an artificial medium composed of a decoction of pea leaves, gelatine (7 percent), asparagine (.25 percent), and saccharose (.5 percent). He named the organism *Bacillus radicicola,* altho he described an organism bearing a single polar flagellum. This organism became generally described as *Pseudomonas radicicola,* and some writers still prefer this designation. The organism has been known under a variety of terms, as *Schinzia leguminosarum, Cladochitrium tuberculorum, Rhizobium radicicola, Rhizobium leguminosarum, Bacterium radicicola, Micrococcus tuberigenus, Myxobacteriaceae, Actinomyces,* and *Phytomoyxa.* Such inappropriate names as *Rhizobacterium japonicum* and *Rhizobium sphaeroides* are applied to certain special races. In commercial and general use, the organism is labeled as pea bacteria, bean bacteria, alfalfa bacteria, et cetera.

Recent studies on the number of flagella possessed by this organism have indicated that the organism is a bacillus, and it is therefore desirable to adopt the original name *B. radicicola,* as proposed by Beyerinck.

BACILLUS RADICICOLA

These bacilli are rod-shaped organisms possessing numerous flagella (6 to 20), which are peritrichous. When full grown they vary in length from 1 to 4 or 5μ.[2] It is not uncommon to find them from .5 to $.6\mu$ wide and from 2 to 3μ long, and some have been found to measure only $.18\mu$ wide and $.9\mu$ long. The organism is actively motile. It is strongly aerobic, and in this connection Pierce calls attention to the intracellular spaces in the root, which make it unnecessary to assume, as has been done, that it must live anaerobically. It is known that this organism does not form spores, but its means of enduring in the soil has not yet been determined. The bacilli prevail in the young nodule, while the branched forms, or bacteroids (see page 487), predominate in the older structure.

Fig. 5.—B. radicicola, showing shape and flagella

B. radicicola grows well on a great variety of culture media, perhaps best on a medium of ash-maltose-agar or one of legume extract plus a sugar and dipotassium phosphate. Dextrose, saccharose, and maltose are suitable carbohydrates. The cultural characteristics of the colonies and the morphology of the organism will not be considered at this time, but it might be stated that many modifications occur on various media.

[1]Beyerinck: Bot. Ztg. (1888), **46**, 725; also 741, 757, 780, 797.
[2]μ=micron, or 1/25,000 of an inch.

GROWTH AND ENDURANCE OF B. RADICICOLA

The optimum temperature for *B. radicicola* varies between 18° and 26°C. The thermal death point, according to Zipfel,[1] is 60° to 62° C. Growth is perceptible between 3° and 46° C.

B. radicicola is not very sensitive to the reaction of the medium, which may be either acid or alkaline. Under field conditions, the organism exists in extremely acid soils, especially the race peculiar to legumes which thrive well on very acid soils. Experiments have demonstrated that the bacteria can withstand any degree of acidity or of alkalinity in the soil, that the particular legume itself can endure.

That the organism endures at least two years in dry soil was determined by Ball.[2] Harrison and Barlow[3] found that the limit of viability on ash-maltose-agar varied somewhat, but that in the majority of cases it was about two years. No doubt the organism will live much longer than this on artificial media when suitable conditions of growth are maintained. How long the clover or alfalfa organism will exist in a soil under field conditions is not yet known, but practical observations indicate that it must be many years.

The statement is quite generally made that *B. radicicola* becomes "nitrogen hungry" when cultivated thru several generations on nitrogen-free media. This fact has not been sufficiently demonstrated to be accepted, for while Süchtung, Hiltner, and others have found that these organisms survive cultivation on nitrogen-free media for a year and at the end of that time possess the same ability to effect inoculation and nitrogen fixation in the legume as organisms obtained from fresh nodules, yet the bacteria had apparently made no appreciable gain in ability to effect inoculation. Garman and Didlake[4] failed to find that nitrogen-free medium possessed any particular advantage over a legume-extract medium in causing the organism to become "nitrogen hungry."

INDENTITY OF B. RADICICOLA

It is believed by some that the various legumes have different species of bacteria. Evidence has been produced which indicates that nodules do not contain but a single race of infecting organisms. Gino de Rossi[5] reported the finding, in artificial cultures, of two organisms which differed in that one formed a large hyaline colony, not developing well in beef and peptone gelatine, while the other

[1]Zipfel: Centbl. f. Bakt. 2 Abt. (1912), 32, 97-137. Five minutes taken as the time of exposure instead of ten minutes.

[2]Ball: Centbl. f. Bakt. 2 Abt. (1909), 23, 50.

[3]Harrison and Barlow: Centbl. f. Bakt. 2 Abt. (1907), 19, 429.

[4]Garman and Didlake: Ky. Agr. Exp. Sta. Bul. 184, 352.

[5]Gino de Rossi: Centbl. f. Bakt. (1907), 18, 289-314, 418-489.

formed white non-transparent colonies in beef gelatine. He believed that he had found another organism associated with *B. radicicola*. This work has not been sufficiently substantiated to be accepted as final. Greig-Smith[1] reported having found three races of this organism in the same nodule. Hiltner and Störmer[2] classify nodule bacteria into two groups, *Rhizobium radicicola* and *Rhizobium beyer-inckii*. The former they associate with lupines, serradella, and soy-beans; the latter with all other legumes.

On the other hand, the results of many investigators[3] (especially Laurent,[4] who obtained nodules on the pea with organisms from thirty-six different legumes, and Nobbe et al.,[5] who worked on the adaptability of nodule bacteria of unlike origin in different genera of Leguminosae), seem to support the theories of the identity of nodule organisms and the presence of only one race in the nodule.

On the whole, present experimental evidence is slightly in favor of the view that there is only one species of this organism thruout the entire family of legumes.[6] This conception is not easily reconciled with field observations, for under natural conditions this organism has become so modified as to make it appear that there are many species. Contamination of the nodule has undoubtedly been responsible for varying conclusions in this connection.

Enzyme Production by B. radicicola

Hiltner[7] reported the finding, by filtration thru porcelain, of a substance produced by *B. radicicola* which can dissolve the cell wall of root hairs. No proteolytic enzyme has as yet been reported. Further, Beyerinck claims that no enzyme has been found which attacks lime, starch, or cellulose, or which is capable of inverting saccharose. In recent studies, Fred,[8] altho unable to detect a proteolytic enzyme, obtained slight evidence of the presence of oxidases in the slime of various legume bacteria. These results suggest the need of further studies on enzyme production by this organism. Similar to all microörganisms, it has the ability to reduce methylene blue to the colorless leuco-compound.

[1]Greig–Smith: Jour. Soc. Chem. Indus. (1902), **26**, 304–306.

[2]Hiltner and Störmer: Arb. K. Gsndhtsamt, Biol. Abt. (1903), 3H. **3**, 151.

[3]Harrison and Barlow: Centbl. 2 Abt. (1907), **19**, 429.
Kellerman: Centbl. f. Bakt. 2 Abt. (1912), **34**, 45.
Buchanan: Centbl. f. Bakt. 2 Abt. (1909), **22**, 371.

[4]Laurent: Exp. Sta. Rec. (1890), **2**, 186.

[5]Nobbe et al.: Centbl. f. Bakt. 2 Abt. (1895), **1**, 199.
   ,,   ,, ,, : Ibid (1900), **6**, 449–457.

[6]Kellerman (see note 3) reported inoculation of soybean, lupine, and also of alfalfa from a culture originally isolated from alfalfa and kept on artificial media in the laboratory for six years.

[7]Hiltner: Centbl. f. Bakt. 2 Abt. (1900), **6**, 273.

[8]Fred: Vir. Agr. Exp. Sta. Ann. Rpt. 1910–11, 123.

SLIME PRODUCTION BY B. RADICICOLA

B. radicicola produces, on artificial media, a gum, or slime, which is partly soluble and party exists as a zoogloeal mass. The organisms, on suitable media, have been observed to surround themselves with definite capsules several times thicker than themselves. These capsules are rather distinct at first but later form a gelatinous mass. Greig-Smith[1] and Mazé,[2] who studied this slime, claimed for it a nitrogenous substance. The results obtained by Buchanan, Gage, and Fred agree and are in direct refutation of the above. This is important to bear in mind in connection with the theories later to be discussed. Gum is not formed from a carbohydrate containing less than five carbon atoms.

ISOLATION OF B. RADICICOLA FROM SOIL

Until recently *B. radicicola* had never been very successfully isolated from a normal soil except by means of a legume plant. Gage,[3] after a long, tedious process, obtained from soil an organism which was capable of producing nodules on red clover and which appeared to be identical with *B. radicicola*. Still more recently Greig-Smith[4] has reported the isolation of this organism from soil, but his work has not been substantiated by others.

Mazé early attempted the isolation of *B. radicicola* from sterilized and non-sterilized soils to which pure cultures had been added. He isolated the organism from the soil which had been sterilized before the addition of the culture, but he was unable to recover it from the unsterilized soil. Kellerman and Leonard[5] isolated (on the agar recommended by Greig-Smith) an organism which inoculated alfalfa from soil that had been sterilized and subsequently inoculated with living organisms of *B. radicicola*. Lipman and Fowler[6] were able to isolate the organism peculiar to vetch (*Vicia sicula*) on soil-extract agar and proved out the organism. They attained success in about 40 percent of the cases, judging from the condensed report recently published.

DISSEMINATION OF B. RADICICOLA

Those familiar with pot-culture experiments and inoculation experiments with legumes easily understand that legume bacteria are disseminated in many ways. In fact, sterile conditions are difficult to maintain. The layman, however, may wonder how legume bacteria

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[1]Greig–Smith: Centbl. f. Bakt. 2 Abt. (1911), 30, 552–556.
[2]Mazé: Ann. Inst. Pasteur (1898), 12, 128.
[3]Gage: Centbl. f. Bakt. 2 Abt. (1910), 27, 7–48.
[4]Greig–Smith: Centbl. f. Bakt. 2 Abt. (1912), 34, 227–229.
[5]Kellerman and Leonard: Science (1913), 38, 95–98.
[6]Lipman and Fowler: Science (1915), 41, No. 1050, 256–258.

not common to a certain locality creep in. A few agents concerned in the transfer of the organisms are here cited.

The seeds themselves are a common means of distribution. The ruptured seed coats offer an opportunity for the various kinds of bacteria to accompany the seeds in a most persistent manner. Sometimes wind is responsible for the dissemination of these bacteria. An interesting instance is cited by Ball of Texas: a wind storm blew off the roof of the culture-house in which legumes were being grown under sterile conditions, and as a consequence the various plants under observation became inoculated. Water has been known to aid in inoculating large areas during washing and floods. The transfer of uncleaned seed is sure to result in the conveyance of some inoculating organisms in the impurities accompanying the seed. Cultivation, especially harrowing, is also responsible for the spread of the organisms. The addition to soils of legume residues from either fields or stables is still another common means of dissemination.

FIXATION OF NITROGEN WITHOUT THE LEGUME PLANT

Mazé,[1] in 1897, demonstrated that nodule bacteria have the power to assimilate atmospheric nitrogen in the absence of a legume. His researches have been verified by others, altho the amounts of nitrogen obtained in his experiments have never been equaled. Recently conducted experiments[2] on this question show that as an average, in liquid and in solid media, about 1.2 milligrams of nitrogen are fixed per 100 cc. of medium. A fixation in the absence of the legume plant has been found in sterile sand and in soil. How important this kind of non-symbiotic fixation is, has yet to be more fully determined. At the present time it is generally recognized as insignificant compared with symbiotic fixation in the nodules of legumes.

BACTEROIDS

Bacteroids are believed to be a form which appears in the development of *B. radicicola*. The cell activities of this form are maintained in a way similar to that in which the cell activities of the rod-shaped form are maintained. Stefan[3] states that these bacteroids are thin-walled and capable of division when young, but that when older they become swollen and finally degenerate. Fred was able to observe the changes which occur in the organism in passing from the bacillus to the extreme vacuolized bacteroidal form. The organism at first apparently thickens at one end and then branches into the bacteroid, which is characterized by rounded outgrowths, known as vacuoles. These vacuoles appear at a definite period of growth and evidently

[1]Mazé: Ann. Inst. Pasteur (1897), 11, 44.
[2]Fred: Vir. Agr. Exp. Sta. Ann. Rpt. 1909–10, 138–142.
[3]Stefan: Centbl. f. Bakt. 2 Abt. (1906), 16, 131–149.

are not a sign of polymorphism, but are a further development of the bacteroid. (They require special staining to be made visible.)

Fig. 6.—Bacteroids, showing shape, and occurrence of vacuoles

Bacteroids occur in the nodule as well as on culture media. Their morphology varies according to the constituents of the culture media. Some writers prefer to call these irregular organisms degenerate or involution forms. They were first observed in artificial media in 1888 by Beyerinck,[1] and have been studied by Hiltner,[2] Stutzer,[3] Buchanan,[4] Fred,[5] and others. A medium rich in carbohydrates or the glucosides of amygdalin or salicin offers very favorable conditions for bacteroid formation. Of fifteen carbohydrates tested, mannite has proved particularly suited to their development. Glycerine is better than most nutrients, while the salts of organic acids have been found unsuitable.

On careful observation the following factors have been found to exercise no influence upon bacteroid formation: temperature, light, osmotic pressure, decreased oxygen pressure, reaction of medium, nitrogen-hunger, specific formative materials in the legumes, and the accumulation of metabolic products. From the above observation it is evident that nutrition is a strong factor in bacteroid formation.

It is claimed that each of the various legumes exhibits a different shaped bacteroid which is characteristic of that legume. In studies conducted at the Virginia Experiment Station the bacteroids possessed by the Egyptian, the crimson, and the red clover were found to be very similar, while those of the vetch differed somewhat. More extended research regarding the appearance of bacteroids as connected with the beginning of nitrogen assimilation, is sorely needed.

THEORIES OF ASSIMILATION, FIXATION, AND IMMUNITY

THEORIES OF ASSIMILATION BY THE PLANT

In brief it may be said that the two main suppositions regarding assimilation are as follows: (1) that the bacteroids are bodily absorbed by the plant fluids; and (2) that the bacteroids, by some sort of change, produce the substance containing the assimilable nitrogen which the plant utilizes.

The theory that the plant absorbs these bacteroids has been challenged by some on the evidence which Nobbe and Hiltner[6] produced

[1] Beyerinck: Bot. Ztg. (1888), **46**, 725.
[2] Hiltner: Centbl. f. Bakt. 2 Abt. (1900), **6**, 273.
[3] Stutzer: Ibid (1901), **7**, 897.
[4] Buchanan: Ibid. (1909), **23**, 59–91.
[5] Fred: Vir. Exp. Sta. Ann. Rpt. 1909–10, 128–198.
[6] Nobbe and Hiltner: Centbl. f. Bakt. 2 Abt. (1900), **6**, 449.

to show that a plant had fixed 1 gram of nitrogen while its nodules weighed only .3 gram. The relationship between the amount of nitrogen fixed and the weight of the nodules is no criterion, however, for criticism of such a theory, inasmuch as this relationship is not at all definite but varies according to the development and needs of the plant. The failure to establish the presence of a proteolytic enzyme has also been responsible for no little criticism of the first supposition.

While the second supposition seems more plausible, it must be admitted that it, too, is only a theory which should be studied with the hope of isolating and identifying the diffusible substance. In connection with this theory Golding[1] conducted some very interesting experiments on the removal of the products of growth in the assimilation of nitrogen by legume bacteria. He reasoned that the plant played an important rôle in the removal of the products produced by bacteria in the nodule aside from the mere furnishing of suitable food. In his experiments he used a porous Chamberland filter-candle placed in a culture vessel to serve to imitate natural conditions. Aerobic conditions were obtained by passing purified air thru the cultures. The parts of the plants used in some of his experiments were sterilized in order to avoid the possibility of plant enzyme action. As a result of his method of experimentation, he obtained a much greater fixation of nitrogen than other experimenters had found, and the logical conclusion arose that the plant performs a function in the assimilation of nitrogen which is construed to be the removal of soluble products of growth. The results of Golding's most extensive experiment are embodied in the table below:

	N. in grams
500.0 grams of Stems and Leaves	2.865
20.2 grams of Roots and Nodules (quite fresh)	.094
3000.0 cc. Ammonia–free Distilled water	.000
Total Nitrogen to start with	2.959
2870.0 cc. Filtrates and Drainings	.731
566.2 grams of Wet Residue	2.570
Total Nitrogen after experiment	3.301
Total Gain of Nitrogen during experiment	.342

Theories Regarding the Chemical Phenomena of Fixation

As yet, purely chemical theories of fixation are entirely hypothetical; however, they deserve consideration, for even theories unsupported by facts may have a value in stimulating thought and assisting in the development of more rational views.

Frank,[2] a prominent Frenchman, was among the first to attempt

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[1]Golding: Jour. Agr. Sci. (1905), 1, 59–64.

[2]Frank: Landw. Jahrb. (1888), 17, 504–518; 19, 564.

an explanation of how the plant actually obtains nitrogen. He believed that it came in thru the leaves, and even recently some isolated statements hold to this idea. His view was allied with the conception of stimulation which many held; namely, that the organisms on the root stimulated the plant to fix nitrogen in its leaves. Stocklasa,[1] as a result of his chemical investigations, also believed that assimilation took place thru the leaves,—that amides were first formed, and that these, migrating to the nodules, reacted with glucose and produced protein, which served as the nutrient medium for the bacteria. In this connection he advanced the idea that the bacteria produced an enzyme which enabled the plant to effect this fixation.

Loew and Aso[2] in 1908 suggested that ammonium nitrite was the first compound produced, the nitrous acid being readily reduced to ammonia. Little evidence has been obtained to support this theory; no evidence whatever has been found under controlled conditions.

Gautier and Drouin[3] suggested that the nitrogen is oxidized to nitric and nitrous acid. Winogradsky[4] has advanced the idea that the free nitrogen in the plasma of the organism may unite with nascent hydrogen and form ammonia, which by oxidation would become assimilable. In connection with the two latter theories, it should be emphasized that the presence of nitrites, nitrates, or ammonia in the nodules, roots, or tops of legumes, inoculated or uninoculated, when grown in the entire absence of combined nitrogen, has not been established.

Gerlach and Vogel[5] have investigated non-symbiotic nitrogen fixation and have arrived at the conclusion that there is a direct union of free nitrogen with some organic compound inside the bacterial cell. Heinze[6] thinks it probable that nitrogen is at once brought into combination with a hydrocarbon (glycogen), and suggests that a salt of carbamic acid may be formed first or that carbamic acid may be produced from cyanamid.

There is yet another most extraordinary theory which, owing to its somewhat recent notoriety, it seems appropriate to consider. This theory is most properly called the Jamieson theory. The rather peculiar views embodied in it are perhaps quite well explained in an article published in *The Spokesman Review*, Spokane, Washington, March 28, 1913. *The Review* is a bi-weekly paper devoted to agricultural interests.

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[1]Stocklasa: Landw. Jahrb. (1895), **24**, 827–863.

[2]Loew and Aso: Bul. Col. Agr. Tokyo Imp. Univ. (1908), **7**, 567.

[3]Gautier and Drouin: Bul. Soc. Chim. Paris **78**, 84–97.

[4]Winogradsky: Centbl. f. Bakt. 2 Abt. (1901), **7**, 842.

[5]Gerlach and Vogel: Centbl. f. Bakt. 2 Abt. (1902), **9**, 817–821, 881–892; (1903) **10**, 636–644.

[6]Heinze: Landw. Jahrb. (1906), **35**, 907.

.   "Do Plants Directly Absorb Free Nitrogen from the Air?
"Scotch Scientist is at Odds with the Common Belief

"The doctrine that plants directly absorb free nitrogen from the air conflicts with the earlier beliefs. It is held that plants, with the exception of legumes, cannot utilize the nitrogen of the air, the explanation in the case of legumes being that by the aid of nitrogen in organisms on their roots these plants utilize atmospheric nitrogen.

"Thomas Jamieson, Director of the Agricultural Research Association of Scotland, takes issue with this belief. Mr. Jamieson has made a life study of the problems of plant nutrition. An abstract of his views is given in the New Zealand Journal of Agriculture. Mr. Jamieson proceeds to show:

"1.   That the legume-tubercular theory is untenable.

"2.   That the nitrogen of the air is directly used.

"3.   That the application of this knowledge is valuable to the agriculturalist **********.

"Mr. Jamieson disagrees with the theory that the tubercle formation on leguminous plants is a normal growth containing a net structure of plant food thru the union of fungus and a legume. He says:

" 'I regard the tubercles as abnormal growths. I hold that no "symbiotic" action takes place; that the fungus is not a fixer of the nitrogen; that the legume plant is itself the fixer, and that it sends its manufactured albuminous products to heal up the wound or to counteract the drain of the parasitic fungus; and that the tubercle has nothing to do with the fixation of the nitrogen of the air. ****************'

"As to the explanation of tubercles of leguminous plants Mr. Jamieson says:

" 'The plant being attacked by the fungus, a wound is made, the fluid of the plant courses to repair it, and not only is the leguminous fluid of the plant rich in nitrogen, but its most nitrogenous fluid, albumen, is just a plastic material like the white of an egg, especially suited to heal the wound and to form a sac round the invader.

" 'There is nothing exceptional in the bearing of tubercles by the legume. The nodules, or tubercles, are well displayed. The legume is a plant specially sought by fungus demanding nitrogen. It is provided with a means of supplying the element, hence it is specially attacked.'

"Further investigations lead Mr. Jamieson to the conclusion that nature provides special means for all plants to absorb nitrogen. Even the hardest leaves are soft in the earlier stage. The cultivated members of the legume family have broad, soft leaves studded with apertures, supposed to serve for exhalation. It is accepted that the green cells or the chlorophyl contained by these cells decompose the carbonic-acid gas. Cannot a similar action extend to nitrogen? ****************

"The effect on the soil of producing certain crops, as cereals and grasses, is to reduce the available plant foods. Of these, in their simple forms, the most important supplied by fertilizers are nitrogen, phosphorus, and potash, and of these the farmer can avoid the expense of the purchase of nitrogenous manures by the adoption of a rotation to include those plants that are rich in nitrogen. This is not new. Legumes have been availed of from the earliest recorded time as preparatory to cereals. What is new is that there is a wider field of plants for selection and the farmer knows why these plants enrich the soil for the nitrogen-demanding cereals. The plants, among others mentioned by Mr. Jamieson, are rape, mustard, and turnips."

The above theory has received more contradiction and less support than the others reviewed.[1]

[1]Henry: Ann. Sci. Agron. (1909), **26**, 102–130.
Vageler: Centbl. f. Bakt. 2 Abt. (1909), **22**, 452.
Kövessi: Compt. Rend. Acad. Sci. (1909), **149**, 56.
Kny: Ber. deut. Bot. Gesell. (1909), **27**, 532.
Mameli and Pollacci: Ann. Sci. Agron. (1914), **31**, 141.

Three possible chemical processes in fixation have been considered by scientists:

1. Reduction
2. Oxidation
3. Direct union into an organic compound

The first two possible processes have received no chemical verification, while the third is supported only by data which are of an eliminative character. More data of a similar nature will be found in Part II of the experimental section of this bulletin.

It is interesting to note that opinion seems to be strengthening in support of the theory of the direct union of nitrogen gas into organic combination, in spite of the fact that such a combination is unknown in chemistry to take place at ordinary temperatures. It is possible, however, to unite nitrogen into organic combination at atmospheric pressure, altho a high temperature is required.

THEORIES OF IMMUNITY

Reasoning from animal life, it seems logical for one to believe that the relative strength of a legume plant or of *B. radicicola* may vary under certain conditions so that the plant will resist the entrance of the organism. Inoculation experiments have produced data showing that *B. radicicola* causes a certain resistance on the part of the plant, making it necessary in some cases to employ organisms of greater efficiency in order to produce inoculation.

Hiltner[1] has given the six following conditions as instances in which immunity demonstrates itself.

1. The organisms cannot get into the plant.
2. The organisms gain admission into the plant but do not produce nodules because the plant, by its greater resistance, absorbs the bacteria.
3. The organisms enter the plant and produce nodules, but no fixation of nitrogen occurs.
4. The organisms enter, produce nodules, and nitrogen is fixed and assimilated by the plant.
5. The organisms are so efficient in comparison with the plant that the latter is injured.
6.. The organisms are parasitic and the plant is actually killed.

In the pursuance of the investigations reported in this bulletin no indication of the existence of any of these conditions, except No. 4, was observed, and in no instance under normal conditions did inoculation fail to produce nodules and cause a fixation and an assimilation of nitrogen.

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[1]Lafar: Handbuch der technischen Mykologie (1904–6), 3, 45.

Various other similar theories of resistance have been proposed, all of which are permeated with the idea of natural or acquired immunity. Prominent among these might be mentioned Süchtung's theory of equilibrium.[1] Süchtung assumed that the bacteria produced a toxin and the plant an antitoxin, and that the degree of equilibrium determined the extent of nodule formation, the plant becoming immunized by an antibody and not by a substance produced by the bacteria; further, that the nitrogen supply in the plant was regulated by the production of this antibody. It is conceivable that in some of the cases observed the apparent immunity may have been due to a weakness on the part of the bacteria rather than to resistance by the plant. Süchtung's equilibrium theory considers varying conditions of virulence on the part of the bacteria and varying degrees of resisting ability by the plants. His theory was advanced as the result of carefully conducted experiments which showed that there were variations in virulence between organisms of the same kind when grown upon artificial media and when obtained from fresh nodules.

The inoculation of legumes in solution is inhibited by potassium nitrate, tho a convincing explanation of this inhibition has not yet been offered. Some experimenters believe that the immunity of a plant is strengthened by its nitrogen nutrition; others hold that bacteria find another source of nitrogen nutrition in the nitrate and hence do not seek the plant. It has been recently shown, however, that the organism will produce nodules after the concentration of the nitrate has been reduced by the plant, which would tend to show that the immunity of a plant is not strengthened and that the organism is not permanently injured by a solution of potassium nitrate suitable for the plant.

Inoculation under field conditions is no doubt inhibited by physical and antagonistic biological factors, which have been considered only briefly by most investigators.

The subject of immunity in plants has been given little attention thus far, but the increasing number of bacterial diseases in the plant kingdom will undoubtedly lead to research in this direction. It is well known that bacterial diseases of plants are the most difficult to control; the need of investigation in this unexplored field of immunity as an aid in their control is imperative.

PRACTICAL CONSIDERATIONS WITH REGARD TO LEGUME FIXATION

MUTUAL SYMBIOSIS

Mutual symbiosis may be defined as the contiguous association of two or more morphologically distinct organisms not of the same kind,

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[1] Süchtung: Centbl. f. Bakt. 2 Abt. (1904), 11, 377.

resulting in an acquisition of assimilated food substances. It implies that the organisms concerned have the power of independent existence, but that both are benefited by the close association.

The relationship existing between *B. radicicola* and legumes is one of mutual symbiosis. The facts which bear out this belief are too convincing to need explanation. However, some prefer to call the relationship a truly parasitic condition, while others consider it to be parasitic in the beginning and later a true mutual symbiosis. This latter conception would seem to be plausible, yet no exact data have been produced to show that a parasitic condition exists at any stage.[1]

The result of this mutual symbiosis is wonderfully characteristic of nature as well as astounding when one considers the corresponding chemical process, in which the energy expended is so apparent and the temperature required so high.[2] The energy values in the symbiotic fixation of nitrogen by *B. radicicola* and legumes have never been determined. When *B. radicicola* and *Azotobacter* are grown under similar conditions, apart from their respective hosts,[3] less organic carbon per unit of nitrogen fixed is oxidized by *B. radicicola* than by *Azotobacter*. Present knowledge indicates that a very great amount of energy is necessary for the fixation of atmospheric nitrogen by *Azotobacter*.

Table 1 presents the amounts of some of the common materials that must undergo rather complete oxidation in order to furnish sufficient energy for the addition of fifteen pounds of atmospheric nitrogen to the surface soil of an acre by *Azotobacter*. The figures show that in the case of dextrose 66⅔ times as much organic matter is re-

TABLE 1.—AMOUNTS OF MATERIALS NECESSARY FOR THE FIXATION OF FIFTEEN POUNDS OF ATMOSPHERIC NITROGEN PER ACRE BY AZOTOBACTER

Kind of material	Pounds required
Dextrose (sugar)	1 000[1]
Fresh clover tops	1 212
Fresh lupine tops	2 000
Wheat straw	4 300
Corn stover	5 500
Oak leaves	11 500

[1]This figure represents the minimum amount of dextrose consumed per unit of nitrogen fixed; in other words, 1 gram of dextrose (yielding 3,750 calories) is necessary for the fixation of 15 milligrams of atmospheric nitrogen by *Azotobacter*.

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[1]Experiments are now in progress at this station with the view of obtaining data on this question.

[2]The most recent figures show that 1 kilowatt hour yields 70 grams of nitrogen in the form of cyanamid; in other words, 1.35 horse-power yields 70 grams, or 8.74 horse-power per hour yields 1 pound of nitrogen.

[3]Algae are understood as the host for *Azotobacter*. The word *host* as used in this publication is not intended to convey the idea of parasitism.

quired as there is nitrogen fixed, and in the case of oak leaves, 766 times as much. In the oxidation of such large amounts of organic carbon it is easily seen that the volume of organic matter in the soil is greatly reduced.

It has been definitely shown that *Azotobacter* lives in a symbiotic relationship with algae. It is also well known that our normal soils possess an abundant algal flora. In view of these two facts it may possibly be found that nitrogen is accumulated by *Azotobacter* without the above reduction in the volume of the organic matter of the soil.

Whatever the future may disclose, the only fact that now remains to be pointed out is that in the symbiosis between legumes and *B. radicicola*, instead of there being a decrease in the organic matter of a soil, a material increase is bound to result. While fixation progresses, organic matter is being manufactured by the plant, which is later returned to the soil. This process is, then, a constructive one, as compared with the destructive non-symbiotic fixation.

AMOUNT OF NITROGEN FIXED PER ACRE PER YEAR

The amount of nitrogen added to a soil depends in part upon the relative supply of that element in the soluble and decomposable forms, organic as well as inorganic. The poorer the soil the greater the amount of nitrogen that will be fixed, tho in a rich soil in which the nitrogen is not in an available form, large amounts may be fixed. For instance, altho a peat soil contains in one acre some thirty thousand pounds of nitrogen in the surface million pounds (0 to $6\frac{2}{3}$ inches), yet because of a lack of proper organisms in the soil to decompose the organic matter, which resists natural decay and therefore does not readily furnish nitrogen to plants, a legume crop may add large amounts of that element.

Where nitrates are present in large amounts, they are taken up by legumes; but where they are present in only small amounts, as is the case during dry seasons even on the common prairie corn-belt land, atmospheric nitrogen is fixed by legumes. The fixation varies with the seasonal conditions, a hot, moist season being best suited to the summer legumes. Among other factors the kind of legume and the duration of its growing period affect the amount of nitrogen added. The annual legumes must necessarily fix nitrogen much faster than the biennial or perennial legumes. The yield does not necessarily indicate the amount of fixation, as some legumes which yield much less hay and seed than others may have a greater total nitrogen content.

The most reliable data which now exist indicate that two-thirds of the nitrogen in legumes grown on soils of normal productive power is obtained from the air.[1] These figures, contributed by the Illinois

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[1] Hopkins: Ill. Agr. Exp. Sta. Buls. 76 and 94.

Experiment Station, were obtained by analyzing inoculated and unin-oculated legumes from like areas of normal soils, and as a result of pot experiments. Computed by these data, a 3-ton crop of cowpea hay adds 86 pounds of nitrogen per acre, a 25-bushel crop of soybeans with $2\frac{1}{4}$ tons of straw adds 106 pounds, a 4-ton clover crop adds 106 pounds, and a 4-ton alfalfa crop adds 132 pounds.

A nitrogen gain of 200 pounds per acre has been reported by the New Jersey Experiment Station[1] with crimson clover. At the Rhode Island Experiment Station,[2] as a result of a pot-culture experiment, it was found that nitrogen had been added at the rate of 400 pounds per acre per year. This experiment extended over five years, and legumes were grown both in the summer and in the winter. The tops of the summer legumes (cowpeas and soybeans) were removed from the soil, while the winter legume (vetch) was turned back into the soil. It should be noted, however, that an acre of this soil to a depth of $6\frac{2}{3}$ inches contained only a little over three thousand pounds of nitrogen. Moreover, in this experiment optimum conditions were established, and no losses were possible from drainage,—which factors would tend to make these results much higher than would be obtained under field conditions.

In considering soil enrichment by clover, ten years' results of a field experiment at the Experimental Farms, Ottawa, Canada,[3] are important. In this experiment a light, sandy loam with a sandy sub-soil was planted to clover continuously, being reseeded every two years. The clover was cut and left to decay on the land. In ten years the nitrogen content of this soil was doubled. The yearly *gain* of nitrogen was fifty pounds per acre. It was found that from two to three times that amount was added, but that all but fifty pounds was dissipated by bacterial activities and in other natural ways. Analyses of the clover crop also brought out the fact previously mentioned that the amount of nitrogen fixed is influenced in part by the season.

VALUE OF LEGUMES AS NITROGEN RETAINERS

Legumes have a very great value aside from their rôle in the nitrogen-fixing process. It is well known that they require more nitrogen for their growth than other ordinary farm crops, and that they therefore contain more of it per ton. It seems very appropriate, therefore, to select legumes for such purposes as holding soil from

[1]N. J. Agr. Exp. Sta. Rpt. 1894, 158.

[2]R. I. Agr. Exp. Sta. Bul. 152.

[3]Experimental Farms, Ottawa, Rpt. 1912, 145.

washing and preventing sands from shifting, for not only do they serve these purposes well, but at the same time they conserve relatively more nitrates from loss than non-legumes. Of course a condition might occur in which a legume would draw all its nitrogen from the soil, but even in such a case a legume would be preferable to a non-legume, as by its use relatively more nitrogen would be kept from leaching and so saved for future crops.

CROSS-INOCULATION

There are relatively few cases of cross-inoculation that have been definitely determined as occurring under natural conditions. The most important example is the cross-inoculation that takes place between the sweet clovers and alfalfa. Bur clover (*Medicago lupulina*) is another source of inoculation for alfalfa. The wild vetches serve for inoculation of the cultivated vetches. It would seem that many such cases may exist in which the wild specie of a legume contains the organism for the inoculation of the cultivated legume of the same specie or even of an entirely different specie or genera. Investigations along this line have not been carefully undertaken as yet.

It has been possible under laboratory conditions to cross-inoculate in many different ways. The data furnished by Laurent, referred to in an earlier part of this publication (page 485), together with that furnished by Moore, Kellerman and Leonard, and others, is of interest. Laurent produced nodules on the pea with the organisms from thirty-six different species of legumes. Moore[1] produced nodules on many legumes with the pea organism, among which were crimson clover (*Trifolium incarnatum*), white clover (*Trifolium repens*), red clover (*Trifolium pratense*), berseem (*Trifolium Alexandrinum*), alsike (*Trifolium hybridium*), sweet clover (*Melilotus alba*), cowpea (*Vigna catjang*), alfalfa (*Medicago sativa*), broad bean (*Vicia faba*), common bean (*Phaseolus vulgaris*), fenugreek (*Trifolium foenum graecum*), hairy vetch (*Vicia villosa*), scarlet vetch (*Vicia fulgeus*), and yellow vetch (*Vicia lutea*). The results published by Kellerman and Leonard represent the extreme in cross-inoculation at the present time. It will be recalled that they have reported the inoculation of the soybean, the lupine, and alfalfa with an organism originally obtained from alfalfa nodules,[2] altho it has been quite generally believed that the soybean was representative of a special class as regards its inoculation, and the same can be said regarding the lupine. Legumes may be grouped as follows according as their

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[1]Moore: U. S. Dept. Agr. Bur. Plant Indus. Bul. 71.

[2]See page 485, note 6.

bacteria are interchangeable for the purposes of inoculation:

Group 1	Alfalfa, sweet clovers, bur clover, black medick
,, 2	All true clovers
,, 3	Cowpea, partridge pea
,, 4	Soybean
,, 5	Bean
,, 6	Peas (garden and field), vetches (cultivated and wild), sweet peas, lentils
,, 7	Lupines
,, 8	Sanfoin
,, 9	Locust

Nobbe, Hiltner, and Schmid[1] obtained inoculation and nitrogen fixation with the locust and vetch cross-inoculated with each other and with pea bacteria, as shown below:

	Inoculated with	Locust	Vetch	Pea-bacteria
Nitrogen	Locust (Robina)	232.1	13.5	21.2
assimilated	Vetch (Vicia)	12.9	264.0	22.6

Some prefer to divide the legume bacteria into two classes according to the beneficial or detrimental effect produced by lime upon the legume. In this classification, alfalfa would represent one type and serradella the other.

The question of cross-inoculation is far from settled. It is easily seen that a great many interesting problems, aside from the purely scientific studies of the laboratory, are presented for the soil biologist in the pursuance of this field of research.

ASSOCIATIVE GROWTH OF LEGUMES AND NON-LEGUMES

The associative growth of legumes and non-legumes has been given renewed notoriety in recent publications. Practical observations of long standing have indicated that a non-legume benefits by the presence of a legume during the second year of its growth—as might reasonably be anticipated. The proof of a benefit by association during the first season is not sufficiently established for a generalization, for errors in sampling, in methods of experimentation, and other unfavorable conditions have crept in and overshadowed the full value of the data reported.

The problem of associative growth involves many details that must be further studied. The stimulation caused by the struggle for existence in association may increase the height of the crops or the amount of the organic matter produced, yet not necessarily the nitrogen content. In the work under observation at this station, it appears that the nitrogen which is returned to the soil as the nodule sloughs off could hardly be utilized by an ordinary annual non-legume crop. It is yet to be determined whether either the legume itself or its

[1]Nobbe, Hiltner, and Schmid: Landw. Vers. Stat. (1894), **45**, 12.

nodules exude nitrogenous compounds during their active period of growth.

CHEMICAL

The chemical composition of legumes from the standpoint of their nitrogenous constituents has been investigated to some extent, but the studies closely related to this point are relatively few. The following data are very general in character and relate to studies concerning the total nitrogen content of the different parts of legumes at different periods of growth. Studies upon some of the various nitrogenous compounds are also included.

In 1895 Stocklasa,[1] working with lupines (*Lupinus luteus* and *Lupinus augustifolius*), found that the nodules were richest in the element nitrogen at the time of blooming, while the roots appeared to be richest in that element at the fruiting period. His results are given in Table 2. The figures for the nodules indicate that the nitrogen is either taken up by the plant for seed production or diffused into the soil.

TABLE 2.—TOTAL NITROGEN IN LUPINUS LUTEUS: RESULTS OBTAINED
BY STOCKLASA

(Percentage on dry basis)

Period	Roots	Nodules
Blooming.................	1.64	5.22
Fruiting..................	1.84	2.61
Maturity.................	1.42	1.73

Stocklasa also determined protein, amides, and asparagine in lupine nodules. The protein was obtained by the Stutzer method, the amides by the Kjeldahl method, and the asparagine by calculation from the ammonia obtained by distillation with magnesium oxid. Table 3 shows his results.

TABLE 3.—NITROGEN COMPOUNDS IN LUPINE NODULES: RESULTS OBTAINED
BY STOCKLASA

(Percentage on dry basis)

Period	Protein	Amides	Asparagine
Blossoming...........	3.99	.35	.34
Maturity.............	1.54	.15	Trace

The presence of asparagine in the nodule is important, as it is thought to be intimately related with the formation of protein.

In 1901 Wassilieff[2] studied the nitrogen compounds in white lupine (*Lupinus alba*) seeds and seedlings. He found that the seeds contained 7.68 percent of total nitrogen; and that of this, 6.89 percent was in the form of protein and .53 percent was precipitated by phos-

[1]Stocklasa: Landw. Jahrb. (1895), 24, 827–863.
[2]Wassilieff: Landw. Vers. Stat. (1901), 55, 45–77.

photungstic acid, leaving a difference of .26 percent, asparagine. The occurrence of asparagine in large amounts in the seedlings is shown by the data given in Table 4.

TABLE 4.—NITROGEN COMPOUNDS IN FOURTEEN-DAY-OLD GREEN SEEDLINGS OF WHITE LUPINES: RESULTS OBTAINED BY WASSILIEFF

(Expressed in percentage on dry basis)

Parts	P. T. A.[1] nitrogen	Asparagine	Protein	Total nitrogen
Leaves...................	.53	1.45	4.11	6.57
Cotyledons...............	.63	3.83	2.44	7.83
Stems....................	.42	4.57	1.56	6.77
Roots....................	.46	2.20	1.87	5.40

[1]P.T.A.: This abbreviation for phosphotungstic acid will be used thruout this publication.

Wassilieff also demonstrated the presence of leucine and tyrosine in the cotyledons of one-week-old seedlings of white lupines. These and other amino acids would be expected to be present when the protein of the seed is breaking down for the nutrition of the seedling.

Knisely[1] analyzed the leaves, pods, stems, roots, and nodules of lupine plants for total nitrogen at three distinct periods of development. His results show better than the others presented where the nitrogen accumulates as the plant matures.

TABLE 5.—TOTAL NITROGEN IN LUPINES: RESULTS OBTAINED BY KNISELY

(Expressed in percentage on dry basis)

Period	Leaves	Pods	Stems	Roots	Nodules
Full bloom.................	4.02	3.07	1.15	.92	5.17
Pods well formed............	3.70	3.38	.88	.83	4.29
Pods very large.............	3.41	3.68	.90	.66	3.70

Schulze and Barbieri[2] examined lupine and soybeans seeds and seedlings for nitrogen and obtained the results shown in Table 6.

TABLE 6.—NITROGEN IN LUPINE AND SOYBEAN SEEDS AND SEEDLINGS: RESULTS OBTAINED BY SCHULZE AND BARBIERI

(Expressed in percentage on dry basis)

Material	Total nitrogen	Protein	P.T.A. nitrogen	Filtrates from P.T.A.
Lupine seeds..............	8.63	8.17	.24	.22
Soybeans.................	6.73	6.32	.13	.28
Lupine dark seedlings 11 to 12 days old........	10.64	3.40	1.60	5.64
Lupine dark seedlings 12 days old.............	10.51	2.33	2.17	6.01
Soybean seedlings 15 days old.............	7.42	3.86	.56	3.00

[1]Knisely: Ore. Agr. Exp. Sta. Rpt. 1909, 30–31.
[2]Schulze and Barbieri: Landw. Vers. Stat. (1881), 26, 241.

They also found a large amount of asparagine in both the lupine and the soybean seedlings.

Schulze[1] has made a careful study of the compounds in plants, and has formulated the hypothesis that the same decomposition products arise from protein in the plant as outside it, but that in the plant the compounds are further altered, thereby affecting in varying degree the individual products of the hydrolytic decomposition. A comparison of the analyses of pea seedlings one week old and those three weeks old showed the following differences:

	Leucine	Tyrosine	Arginine	Asparagine
1 week..........	abundant	little	present	. absent
3 weeks.........	much less	absent	almost absent	very abundant

Arginine and amido acids were shown to be present in the lupine cotyledons, but asparagine was absent, altho the latter substance was found in the stem of the seedling. It has been suggested that the occurrence of asparagine is associated with the disappearance of amido acids and not of protein. Phenyl alanine, tyrosine, and tryptophane have been reported in the white lupine (*Lupinus alba*), tyrosine and tryptophane in vetch (*Vicia sativa*), and tryptophane in the garden pea (*Pisum sativum*).[2]

Smith and Robinson[3] found 4.19 percent of nitrogen in soybean nodules and 3.90 percent in cowpea nodules. They observed that inoculation increased the protein content of soybean plants without increasing the yield of beans. This has been noted by other experimenters.

Hopkins[4] has reported the analyses of cowpea plants for total nitrogen with and without inoculation. The nodules, roots, and tops were analyzed separately, as will be seen by reference to Table 7.

TABLE 7.—Nitrogen Fixation by Cowpeas: Results Obtained by Hopkins

(Expressed in cgs.)

Treatment	Tops	Roots	Nodules	Nitrogen fixed
Ten plants with bacteria.....·.........	146	9	11	125
Ten plants without bacteria............	38	3	..	...
Ten plants with bacteria...............	171	10	18	140
Ten plants without bacteria...........	55	4	..	...
Ten plants with bacteria...............	143	8	17	124
Ten plants without bacteria...........	40	4	..	...

[1]Schulze: Zeits. f. Physiol. Chem. (1895), 24, 18; 30, 241.

[2]Schulze et al: Zeits. f. Physiol. Chem. (1887), 11, 43; (1906), 48, 387, 396; (1910), 65, 431.
Gorup Besamez: Ber. deut. Chem. Gesell. (1887), 10, 781.

[3]Smith and Robinson: Mich. Agr. Exp. Sta. Bul. 224, 125–132.

[4]Hopkins: Ill. Agr. Exp. Sta. Bul. 94, 319.

The inoculated plants contained a much greater percentage of nitrogen than the uninoculated, the average content of the inoculated being 4.24 percent in the tops, 1.48 percent in the roots, and 5.92 percent in the nodules, while the average content of the uninoculated was 2.48 percent in the tops and .88 percent in the roots.

The ash and the ash constituents of the nodules and the roots of lupines have been determined by Stocklasa,[1] as presented in Table 8. The total ash of the nodules was found to be 6.32 percent, while that of the roots was found to be 4.55 percent.

TABLE 8.—ASH CONSTITUENTS IN LUPINE NODULES AND ROOTS:
RESULTS OBTAINED BY STOCKLASA

(Expressed in percentage)

Constituents	Nodules	Roots
Si...............	1.59	1.90
S...............	4.90	6.38
P...............	6.51	4.28
K...............	17.31	12.05
Na...............	16.94	19.94
Mg...............	7.41	7.05
Ca...............	7.64	12.04
Fe...............	.83	.75

The analyses of red-clover nodules show a potassium content of 2.63 percent in the dry matter.[2] The nodules, therefore, are relatively rich in mineral elements as well as nitrogen compounds; and Stocklasa's results (see Table 8) show that the chief differences between the roots and the nodules in the composition of the ash constituents are in phosphorus, potassium, calcium, and sodium. The nodules are richer in the first two elements and the roots in the latter two. The differences in nitrogen content of the various parts of the plant have already been brought out somewhat, but they will be dealt with more fully in the results presented under the experimental portion of this bulletin. The presence of the bacteria would in itself be sufficient to account for these differences.

In brief, the chemical data which have been considered, altho small in amount, show the relative richness in nitrogen of the nodule as compared with other parts of the plant. They point to the accumulation of nitrogen in the seeds, at the expense of the other parts, as the plant matures. That the nitrogen exists in the form of protein, asparagine, and other soluble forms, is also clear. The presence of various aliphatic and carbocyclic amino acids has been mentioned.

[1]Stocklasa: Landw. Jahrb. (1895), **24**, 827–863.

[2]Analyzed by Aumer, Ill. Agr. Exp. Sta. (unpublished data).

EXPERIMENTAL

Plan of Investigations

The experimental studies herein reported are for convenience divided into two parts. Part I consists of studies made in order to determine thru which organs legumes obtain their nitrogen from the air. Part II is concerned with an attempt to determine more definitely the mechanism of the reactions occurring in the fixation and assimilation of atmospheric nitrogen by *B. radicicola* and legumes, a process concerning which science is greatly in the dark. This phase of the problem has attracted the attention of plant physiologists, physiological chemists, and other scientists outside the field of agricultural research. No lesser chemist than Emil Abderhalden[1] has written concerning it as follows: "It would be very interesting to know the compounds into which these organisms convert the nitrogen. At present we have no knowledge of this. We assume that the final substance produced is protein, which is then in part assimilated by the plants with the help of fermentation." Any light which may be thrown on this question will be of great value toward its final solution.

PART I

STUDIES TO DETERMINE THRU WHICH ORGAN LEGUMES OBTAIN ATMOSPHERIC NITROGEN

For a long time it was believed that the nitrogen fixed by legume bacteria and assimilated by the plant was obtained thru the leaves, and even now many hold to this belief. Frank and Otto[2] in 1890 obtained analytical results which seemed to them to be proof of this theory. They believed that the bacteria were only incidentally connected with the process, acting perhaps as stimuli.

The first experiment resulting in data of a contradictory nature was made by Kossowitsch[3] in 1891, but the results of this investigation were not generally accepted. Nobbe and Hiltner[4] in 1899 added further evidence to the existing knowledge, but their conclusions, drawn from physiological differences, have not been substantiated by chemical data, which seem more reliable than those of a physiological nature.

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[1]Abderhalden: Physiological Chemistry, Trans. by Hall, 198.

[2]Frank and Otto: Ber. deut. Bot. Gesell. (1890), 8, 331.

[3]Kossowitsch: Bot. Ztg. (1892), 50, 697–702, 713–723, 729–738, 745–755, 771–774.

[4]Nobbe and Hiltner: Landw. Vers. Stat. (1899), 52, 455–465.
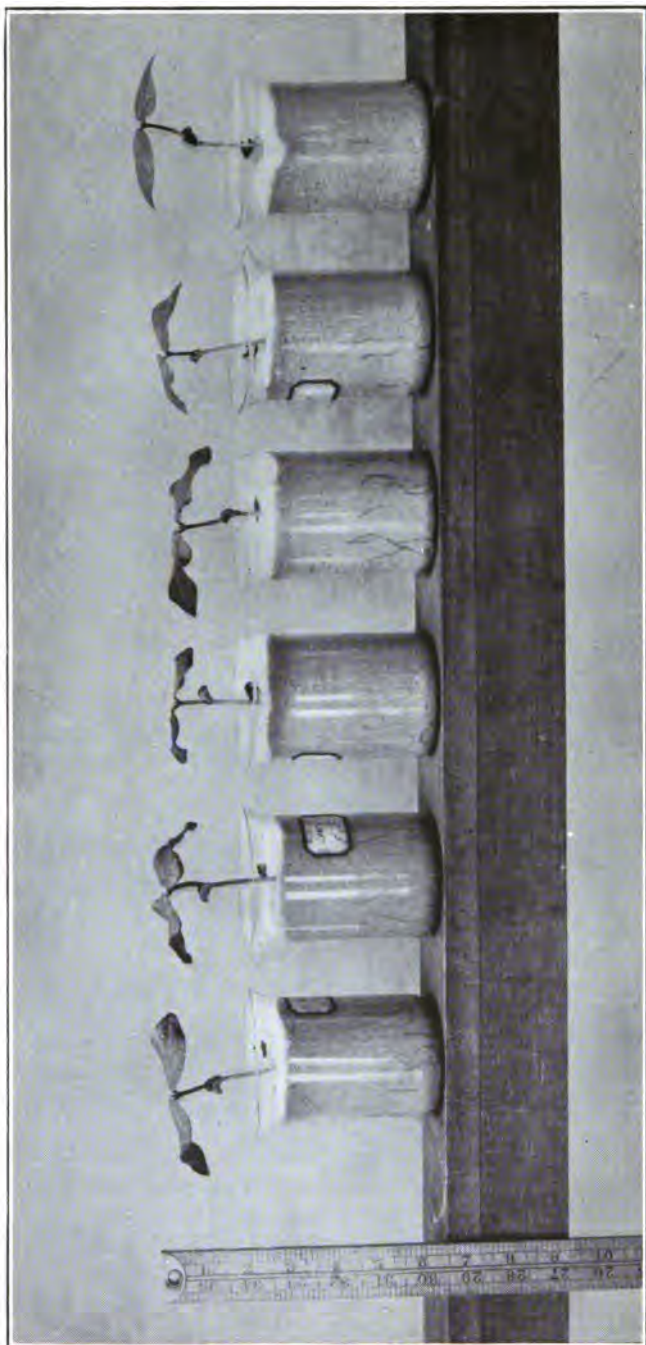
PLATE IV.—COWPEA SEEDLINGS IN PREPARATION FOR GAS EXPERIMENTS

Experiments on this question were conducted by the author in 1911-1912. The general plan was the same thruout each experiment; the various modifications are considered under the individual experiments.

GENERAL PLAN OF EXPERIMENTS

The plants used were the soybean and the cowpea. Uniform seeds were carefully selected and inoculated with an infusion placed directly in contact with them. They were then planted in beakers containing nitrogen-free white sand. Mineral plant food was added in solution. When the seedlings had developed two leaves and possessed small nodules, they were carefully washed from the sand and transferred to the apparatus.

The apparatus[1] was arranged as follows: Woulfe bottles, placed inside battery jars painted black in order to obviate the influence of light, were connected with drier bottles, which in turn were connected with a gasometer. An outlet tube from each bottle was provided, the external end of which was immersed in water. In the first experiment two Woulfe bottles were used and in the others six. Sterile nitrogen-free sand containing calcium carbonate was placed in the Woulfe bottles and the young seedlings carefully transplanted, one to each. The plants were then sealed gas-tight by means of rubber tissue placed double thick about the stem. Rubber cement was also used to make all joints tight.

Plant food, with the exception of nitrogen and calcium, was added in solution. This solution was sterilized, boiled, and cooled just previous to its being used in order to prevent the addition of absorbed gases. The plant-food solutions and sterile, distilled, nitrogen-free water were added from the outer end of the outlet tube, with the gas flowing in order to avoid the possible admittance of air. The moisture content of the sand was maintained at about 12 percent. When the apparatus had been made tight, the gas was started and allowed to flow gently for eight to ten hours per day; at night it was entirely shut off. By this method the plant roots were kept constantly in the same atmosphere.

The gas mixture used in the first three experiments consisted of 96 to 98 percent oxygen and 2 to 4 percent carbon dioxid. For the purpose of comparison, air was passed thru part of the bottles in these experiments. The gas mixture was made in the laboratory, great care being exercised to eliminate nitrogen, air, and other impurities. The oxygen was made from potassium chlorate and manganese dioxid, and the carbon dioxid was generated from marble and hydrochloric acid. The air, when used as a source of nitrogen, was passed thru sulfuric acid before entering the gasometer and after leaving it. In order to

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[1]Plate V shows the apparatus in use.
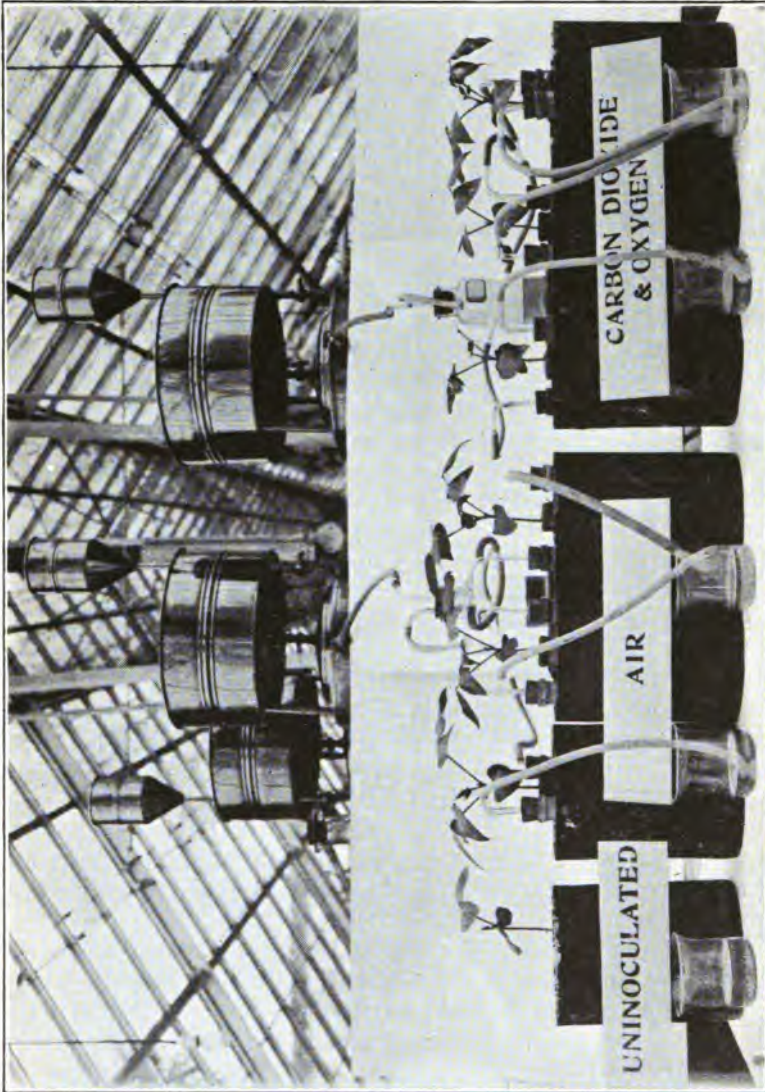
PLATE V.—EXPERIMENT II: COWPEAS AT THE TIME OF HARVEST (37 DAYS)

dispel any possible doubt as to the oxygen mixture being too strong, a fourth experiment was conducted in which the effect of a mixture made of 90 percent oxygen, 7 percent nitrogen, and 3 percent carbon dioxid was compared with that of a mixture made of 97 percent oxygen and 3 percent carbon dioxid.

EXPERIMENT I

In Experiment I, soybeans were used. Three plants twenty-one days old were placed in position on September 1, 1911, one in each of two Woulfe bottles and a check plant left uninclosed. Thruout the experiment these plants were kept out of doors during the day. The experiment was continued for twenty-eight days. At the end of that time the plants were analyzed for total nitrogen by the official Gunning[1] method. The average of individual analyses of twenty soybean seeds was taken as the criterion from which to calculate the amount of nitrogen fixed by the plants. The results are presented in Table 9.

TABLE 9.—FIXATION[1] OF NITROGEN BY SOYBEANS: EXPERIMENT I

(Results expressed in milligrams)

Plant No.	Treatment	Nitrogen in plant at end, 28 days	Nitrogen in check seeds	Nitrogen fixed
1	$CO_2 + O$	10.43	11.4	(−.97)
2	$CO_2 + O$	10.65	11.4	(−.75)
3	Air	17.61	11.4	7.07

[1]The word *fixation* is used in this publication in its broader sense and should be understood as meaning the fixation of atmospheric nitrogen by bacteria and the assimilation of the nitrogenous compounds formed by the plant.

The error in Plants 1 and 2 is partially accounted for by a slight injury to these plants by grasshoppers and red ants. There is, however, a small experimental error which is difficult to eliminate, as will be observed in the other experiments.

EXPERIMENT II

The experience gained in Experiment I led to the selection of cowpeas for the later investigations, since they are less subject to injury by red ants than are soybeans. Experiment II was started on November 23, 1911, and continued until December 29, thirty-seven days. Six two-liter Woulfe bottles were planted with seedlings twenty-four days old. Air was passed thru three of the bottles and the gas mixture thru the other three. The average of individual analyses of

[1]In preliminary tests the Gunning and Kjeldahl methods modified to include nitrates gave no higher results than the official Gunning or Kjeldahl methods.
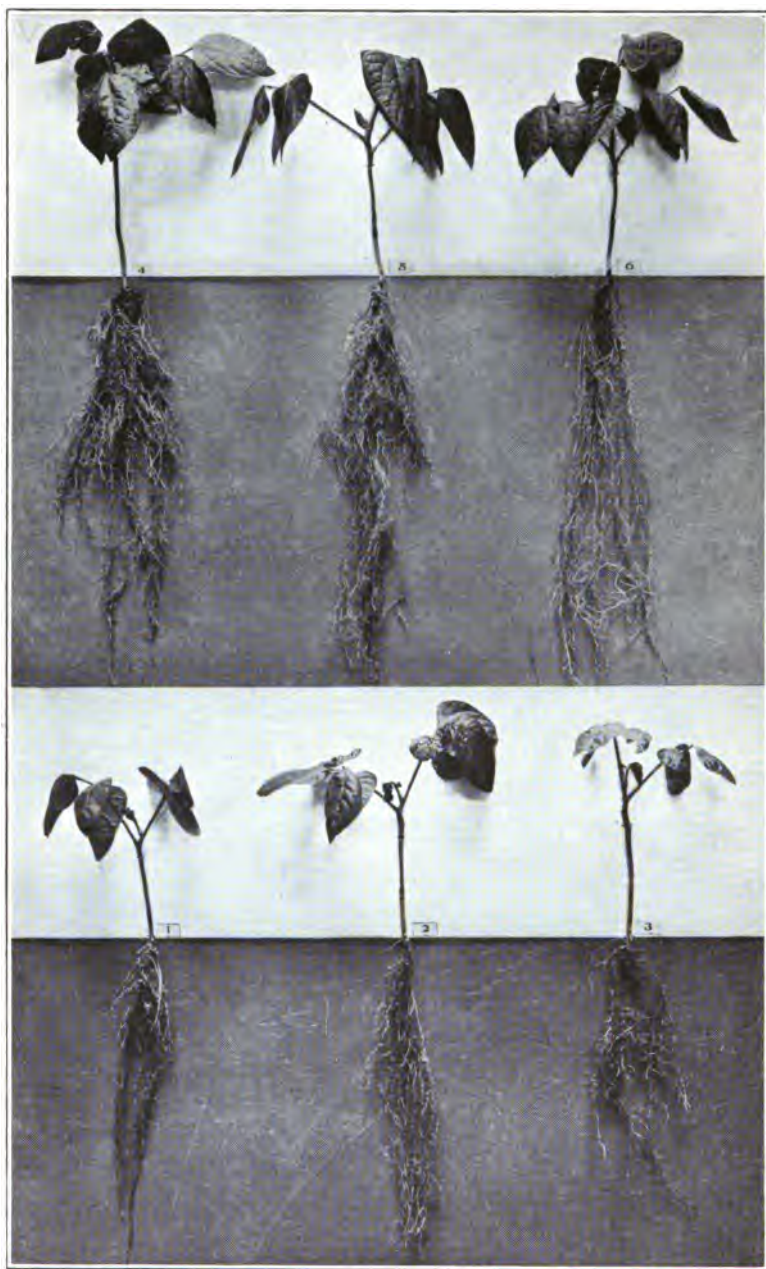
PLATE VI.—EXPERIMENT II: PLANTS ABOVE GROWN IN AIR; THOSE BELOW
GROWN IN $CO_2 + O$

fifteen cowpea seedlings seventeen days old was used as a basis from which to calculate the nitrogen fixed by the plants during the experiment. Seedlings of this age were taken for analysis in order that the results of this experiment might be comparable with those of the others, altho the seedlings of the experiment when transplanted were somewhat older.

TABLE 10.—FIXATION OF NITROGEN BY COWPEAS: EXPERIMENT II

(Results expressed in milligrams)

Plant No.	Treatment	Nitrogen in plant at end, 37 days	Nitrogen in check seedlings at beginning	Nitrogen fixed
1	$CO_2 + O$	9.21	7.90	1.31
2	$CO_2 + O$	13.03	7.90	5.13
3	$CO_2 + O$	9.43	7.90	1.53
4	Air	24.84	7.90	16.94
5	Air	23.61	7.90	15.71

NOTE.—Plant 6 was lost in distilling thru the breaking of the flask, caused by sand adhering to the roots.

The fixation shown by Plant 2 is attributed to a leak discovered around the stem of this plant some few weeks after it had been put in place; trouble was had thruout the experiment in keeping it gas-tight. The evident fixation in the case of Plants 1 and 3 is within experimental error; yet since these plants were twenty-four days old when placed in the apparatus, while the check seedlings analyzed were only seventeen days old, it is reasonable to assume, from the results obtained in the next experiment, that a part at least of the assimilation of this nitrogen had taken place before the seedlings were transferred.

On the plants receiving air the nodules became well developed. The accompanying photograph (Plate VI), taken at the termination of the experiment, shows the comparative development of the roots and the tops grown in the gas mixture and those grown in the air. The most interesting part of this experiment was the very evident translocation exhibited by the plants growing in the mixture of carbon dioxid and oxygen, as shown by their color. The same phenomenon was observed in the later experiments and is discussed under the general consideration of the gas experiments.

EXPERIMENT III

Experiment III was conducted with cowpeas in a manner similar to that of the preceding experiments. It was started on March 5, 1912, with six seedlings seventeen days old and discontinued after eighty-three days, May 27, 1912. Air was passed thru three of the bottles and the gas mixture thru the other three. In order to obtain the best possible check on the results, fifteen additional seedlings of the same
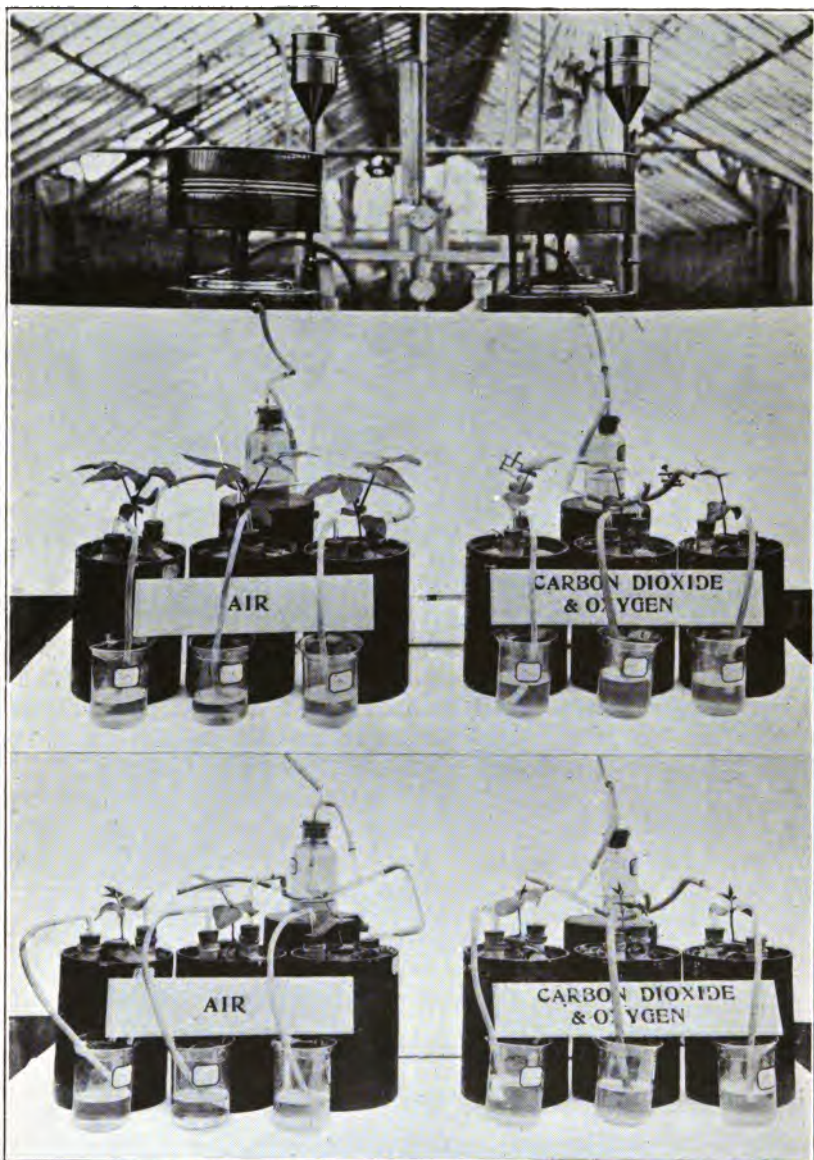
PLATE VII.—EXPERIMENT III: LOWER FIGURE SHOWING EXPERIMENT AT BEGIN-
NING; UPPER FIGURE SHOWING EXPERIMENT 10 DAYS LATER
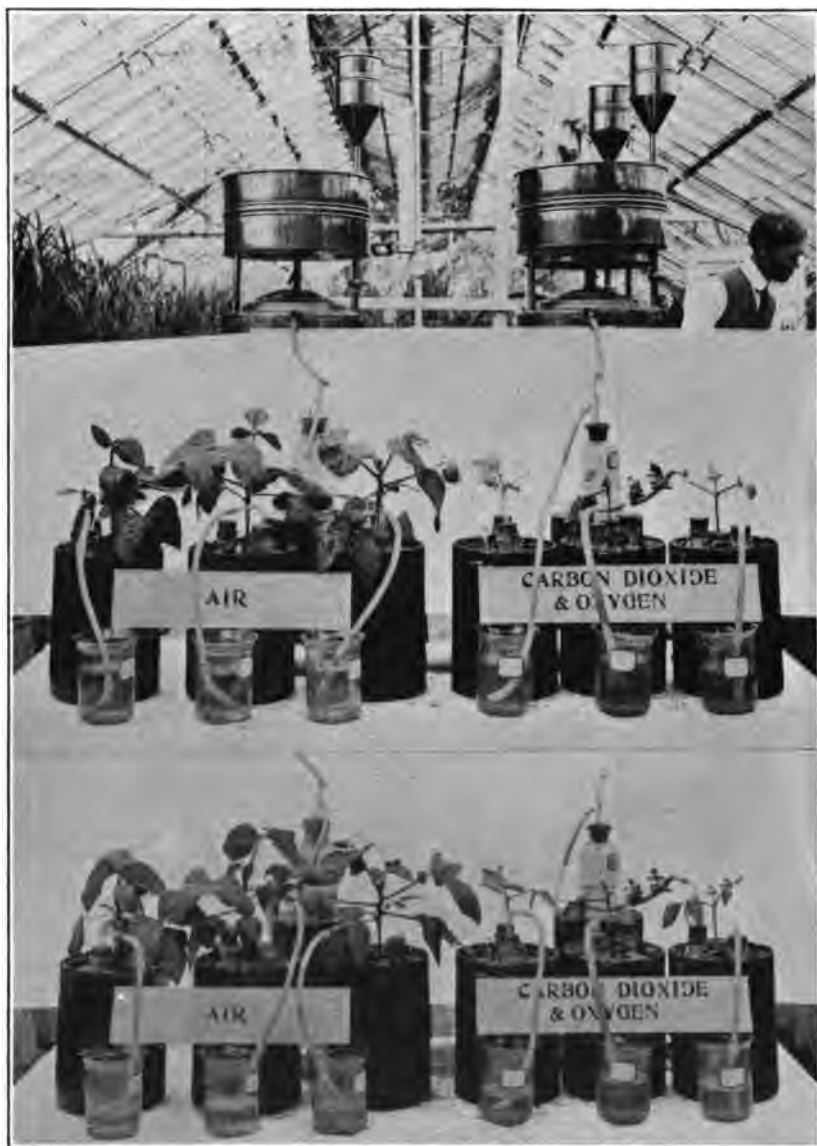
PLATE VIII.—EXPERIMENT III: UPPER FIGURE SHOWING EXPERIMENT 52 DAYS
FROM BEGINNING; LOWER FIGURE SHOWING EXPERIMENT AT HARVEST
(83 DAYS)

lot as those transplanted to the Woulfe bottles, grown from seeds 185 milligrams in weight, were analyzed individually at the beginning of the experiment. The results showed the presence of an average of 7.90 milligrams of nitrogen, while the average nitrogen content of twenty seeds of the same weight analyzed individually equaled 6.94 milligrams, making an average fixation of .96 milligram of nitrogen by these seedlings in the first seventeen days.

TABLE 11.—FIXATION OF NITROGEN BY COWPEAS: EXPERIMENT III

(Results expressed in milligrams)

Plant No.	Treatment	Nitrogen in plant at end, 83 days	Nitrogen in check seedlings at beginning	Nitrogen fixed
1	$CO_2 + O$	9.48	7.90	1.58
2	$CO_2 + O$	7.49	7.90	(−.41)
3	$CO_2 + O$	8.49	7.90	.59
4	Air	Roots 74.27 Tops 112.59 —————— 186.86	7.90	177.96
5	Air	Roots 71.99 Tops 166.03 —————— 238.02	7.90	230.12
6	Air	Roots 66.71 Tops 120.51 —————— 187.22	7.90	179.32

The figures in Table 11 show to what extent fixation took place. Plants 1 and 3 may have contained more than 7.90 milligrams of nitrogen as seedlings, altho it cannot be proved that they did, owing to the impossibility of analyzing and growing the same seedling. There was always another possible source of error in the dissolved nitrogen gas in the water used for pressure in the gasometers.

It is well to observe that in all these experiments the gases were passed thru sulfuric acid, which eliminated the possibility of ammonia playing any part in the fixation. This is claimed by many to occur; yet the first experiment ever made for the purpose of showing that legumes obtain nitrogen from the air was so conducted that combined nitrogen was eliminated.

The plants in the carbon dioxid and oxygen mixture were from 3 to 4 inches in height and possessed two leaves at the end of the experiment, while those growing in the air measured from 8 to 9 inches in height and possessed nine leaves.

PLATE IX.—EXPERIMENT III: ON THE LEFT, ROOTS FROM PLANT GROWN IN CO_2+O; ON THE RIGHT, ROOTS FROM PLANT GROWN IN AIR

In order to test the viability of *B. radicicola* after it had grown on the plant under extreme oxygen conditions, organisms were removed from the nodules of Plants 1, 2, and 3, and an infusion made in sterile water. Portions of this infusion were applied to cowpea seeds that had been sterilized and planted in sterile sand. Sterile conditions were maintained thruout this test. Profuse nodule formation resulted, demonstrating that no harmful results had been produced upon the organism by its long exposure to an atmosphere with a high content of oxygen.

EXPERIMENT IV

Having made certain in Experiment III that no detrimental effects had been produced upon *B. radicicola* by long exposure to an atmosphere high in oxygen, Experiment IV was instituted in order to determine if there could have been any possibility of injury to the plants in the previous experiments from the use of gaseous mixtures high in oxygen.

The plan involved a comparison of the effect of a mixture of 97 percent oxygen and 3 percent carbon dioxid, and that of a mixture of 90 percent oxygen, 7 percent nitrogen, and 3 percent carbon dioxid. The nitrogen used was obtained from the air; otherwise this experiment was similar to Experiment III. Each gas mixture was passed thru three of the Woulfe bottles. The experiment was begun on Sep-

TABLE 12.—FIXATION OF NITROGEN BY COWPEAS: EXPERIMENT IV

(Results expressed in milligrams)

| Plant No.	Treatment	Nitrogen in plant	Nitrogen in check seedlings at beginning	Nitrogen fixed
		First Harvest (26 Days)		
2	$CO_2 + O$	10.00	7.90	2.10
4	$N + CO_2 + O$	14.94	7.90	7.04
		Second Harvest (28 Days)		
3	$CO_2 + O$	8.06	7.90	.16
6	$N + CO_2 + O$	33.51	7.90	25.61
		Third Harvest (95 Days)		
1	$CO_2 + O$	13.97	7.90	6.07
5	$N + CO_2 + O$	Leaves 129.26 Stems 45.17		
		Tops 174.43 Roots 32.93 Nodules 112.02		
		319.38	7.90	311.48
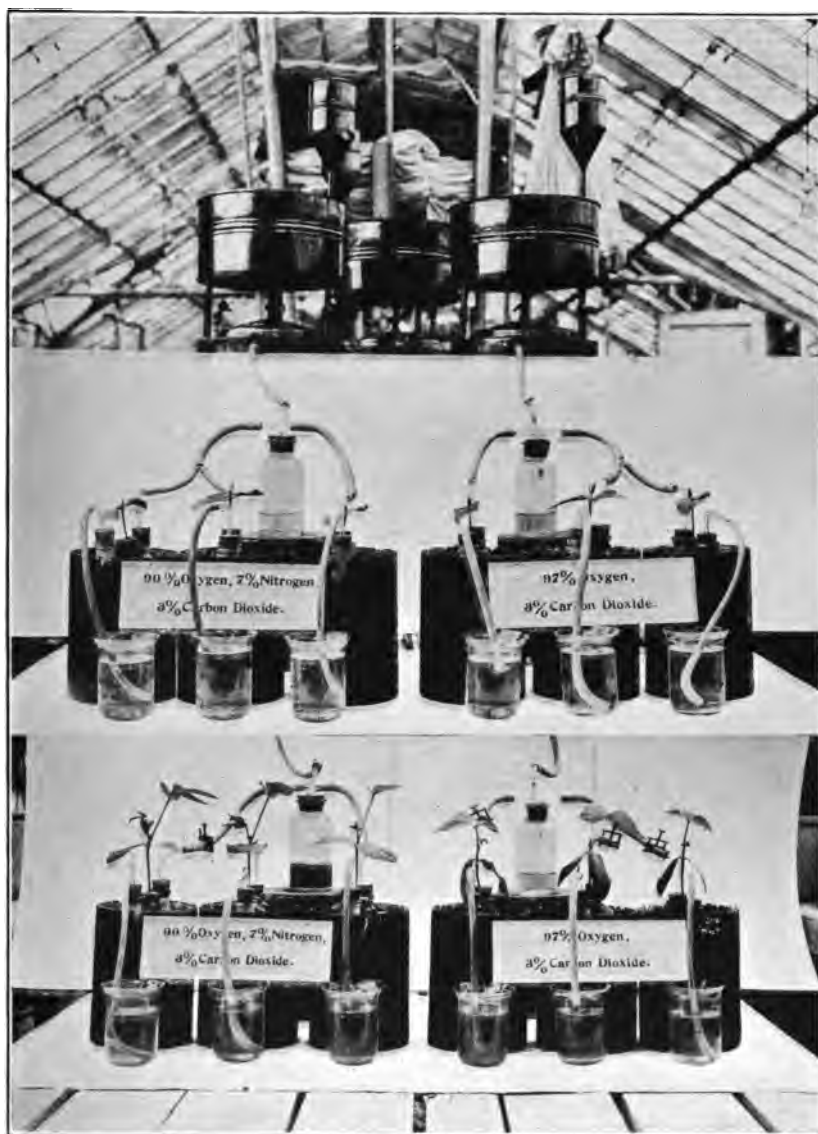
Plate X.—Experiment IV: Upper Figure Showing Experiment at Beginning; Lower Figure Showing Experiment 16 Days Later

PLATE XI.—EXPERIMENT IV: LOWER FIGURE SHOWING PLANTS 41 DAYS FROM
BEGINNING; UPPER FIGURE SHOWING PLANTS 59 DAYS FROM BEGINNING

90 %Oxygen, 7%Nitrogen,   97% Oxygen.
3%Carbon Dioxide.   3% Carbon Dioxide.

PLATE XII.—EXPERIMENT IV: AT THE TIME OF HARVEST (95 DAYS)

PLATE XIII.—EXPERIMENT IV: ON THE LEFT, ROOTS FROM PLANT GROWN IN
CO_2+O; ON THE RIGHT, ROOTS FROM PLANT GROWN IN $N+CO_2+O$

tcmber 7, 1912, with six cowpea seedlings eleven days old, and continued for ninety-five days. The plants were harvested two at each of three periods.

The results given in Table 12 need no explanation, tho it might be well to call attention to the individual differences in the plants in the amounts of nitrogen fixed. During the ninety-five days of the experiment, Plant 5 fixed fifty-one times as much nitrogen as Plant 1. The following comparison between the growth of these two plants is of interest.

Plant 1 attained a total height of 5 inches, possessed one leaf, and on the roots were counted 60 nodules. Plant 5 reached a height of 61 inches; its leaves measured as follows:

2	measured	5	inches	along	midrib,	4	inches	at	base
3	"	4½	"	"	"	3–3½	"	"	"
1	"	4	"	. "	"	3	"	"	"
3	"	3½	"	. "	"	2½	"	"	"
2	"	3	"	"	"	2	"	"	"
3	"	2¾	"	"	"	1½	"	"	"
1	"	3	"	"	"	2	"	"	"

Several pods were formed, as may be seen by reference to Plate XII, one of which measured 4½ inches in length and was partially filled with seeds. The roots were so large that the Woulfe bottle had to be broken in order to obtain them. The plant possessed 32 large nodules, 46 medium to large, 66 medium, and 144 small; 288 in all.

EXPERIMENT BY KOSSOWITSCH

Reference has been made to a laboratory experiment conducted by Kossowitsch in the summer of 1891 (see page 503). As his work has been accepted by some and ignored by others, it is of particular interest.

Peas were started in a mixture of four-fifths sand and one-fifth soil in which peas had been grown the year before. When the plants had developed good nodules, they were transferred to jars containing nitrogen-free sand. In some cases the roots were enclosed and in others the tops, a similar means being used in each case to make the joints air-tight. Over the tops of the jars bell-jars were placed. These were connected thru drier bottles with a gasometer. Because of moisture collecting in the bell-jars, absorbents were used to keep the atmosphere normal. The gas mixtures used were hydrogen and oxygen in some cases; hydrogen, oxygen, and carbon dioxid in some; and air in others. Combined nitrogen was also used to check up the possible abnormal condition due to the necessary manner of experimentation. Great accuracy was displayed in arranging the apparatus and in analyzing the gases.

TABLE 13.—RESULTS OF EXPERIMENT BY KOSSOWITSCH

Plant No.	Duration of experiment	Number of leaves when placed in jar	Part enclosed	Gas used	With or without combined nitrogen	Nitrogen content at end	Nitrogen content of control	Nitrogen fixed
	days					*mgs.*	*mgs.*	*mgs.*
1	65	3	Roots	$H+O$	Without	6	6	(—)
2	65	4	Roots	$H+O$	Without	17	14	3
3	62	4	Roots	$H+O$	With	45	14	31
4	55	4	Roots	$H+O+CO_2$; air later	Without	43	14	29
5	61	5	Leaves	$H+O+CO_2$	Without	26	14	12
6	76	2	Leaves	$H+O+CO_2$	Without·	49	10	39
7	76	2	Leaves	Air	With	35	10	25
8	78	2	Roots	Air	Without	48	10	38
9	30	3	Roots	$H+O$	Without	11	13	(−2)
10	31	3	Roots	$H+O$	With	16	13	3
11	31	3	Roots	Air	Without	20	13	7
12	15	3	Leaves	$H+O+CO_2$	Without	19	13	6

A study of the results of Kossowitsch's experiment, given in Table 13, discloses very little data bearing strictly on the question, thru what organs plants obtain atmospheric nitrogen. Plants 1 and 2 tend to show that nitrogen cannot be obtained thru the leaves. Plant 2 shows a fixation, but it may be within experimental error. Plants 3, 4, 6, and 10 may be eliminated, as assimilation should have taken place in these cases. Plant 5, the leaves of which were in hydrogen, oxygen, and carbon dioxid, fixed nitrogen, but Kossowitsch states that this result is not reliable, owing to the leaves not having been sufficiently isolated. Plants 7, 8, and 11 show normal results, and, like Plant 9, indicate, aside from the experimental error, that the nitrogen must be brought into contact with the roots in order to effect fixation. Plant 12, the leaves of which were enclosed in hydrogen, oxygen, and carbon dioxid, also showed a fixation. Thus it will be seen that the data bearing on the question were obtained from only seven plants.

Somewhat related to this problem is the work of Nobbe and Hiltner,[1] who grew lupines in solutions. They thought that it was necessary to raise the nodules above the solution in order to obtain a fixation, and this seemed to indicate to them that legume plants obtain atmospheric nitrogen thru their roots rather than thru their tops.

GENERAL CONSIDERATION OF GAS EXPERIMENTS

The plants in the mixture of carbon dioxid and oxygen usually developed two and sometimes three leaves before they seemed to be checked in their growth. Soon an interesting translocation set in. Each plant removed the nitrogen from the lower leaves and developed a new leaf of a normal green color. The green of the old leaves disappeared from the margins first; soon the whole leaves became yellow, and shortly dropped from the plant. This process repeated itself until there was not nitrogen enough left in a translocatable form to give green color to another leaf, when a pale green or even a yellow leaf appeared. The duration of this process was remarkably long in some of the plants. The appearance of the plants was in every case of especial note, even to a layman.

It should be emphasized that *no combined nitrogen was present* or could have been assimilated in these experiments. The slight amount of nitrogen reported as fixed by the plants in carbon dioxid may have been either actual fixation or experimental error. From close observation and study made in order to reduce this error, it would seem that its only possible source, disregarding Plant 2 in Experiment II, already mentioned, would be the dissolved air in the water used for pressure in the gasometers. This was unavoidable. It may also account for the error in Kossowitsch's experiment. Plant 1 in Experiment IV

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[1] See page 503, note 4.

would seem to indicate that the above view is correct, as it showed a content of 6 milligrams of nitrogen after having remained under experiment for ninety-five days. In this length of time there were a great many changes of the gasometers and the use of a large amount of water, which would tend to increase the atmospheric nitrogen and thus perhaps contaminate the other gas mixtures. The nodules on all the plants were normal. On Plant 5, Experiment IV, they were very large, and the spongy sutures were highly developed as if to present as large an absorbing surface as possible.

Finally, it should be noted that had the plants growing in the mixture of carbon dioxid and oxygen possessed any ability to take in nitrogen thru their leaves, they should have made as good a development as those growing in the air, and those in the oxygen, nitrogen, and carbon dioxid mixture.

PRACTICAL APPLICATION OF RESULTS

The practical application of the results obtained in these experiments would appear to rest in the proper aeration of the soil in order that greater amounts of nitrogen may be fixed. As the plants obtain their nitrogen thru their roots, it is essential that the soil contain plenty of air at all times.

PART II
RELATIVE PERCENTAGES OF NITROGENOUS COMPOUNDS IN THE VARIOUS PARTS OF THE SOYBEAN AND COWPEA AT DEFINITE PERIODS OF GROWTH

It has seemed advisable to determine, if possible, in what forms atmospheric nitrogen exists in the various parts of the legume, for it is believed that this knowledge would throw much light upon the whole process of fixation. To do this, various compounds in the nodules, roots, and tops were determined at definite periods in the growth of the soybean (*Glycine hispida*, Maxim) and the cowpea (*Vigna unguiculata*, Walp). As it would be quite impossible to determine all these compounds separately, they were grouped and determined as follows,—total, insoluble, and soluble nitrogen. In the soluble-nitrogen group was included the nitrogen precipitated by P.T.A. and Other nitrogen. The constructive and destructive metabolisms in the plant doubtless give rise to other nitrogen compounds than pure protein; so it is quite possible that in addition, proteoses, peptones, peptides, acid amides, amino acids (mono and di), guandine residues, pigment nitrogen, alkaloids, ammonia, nitrites, nitrates, and other nitrogen compounds may have been present.

Because of the great importance of controlling conditions under which experimental plants are grown, all the factors in this work were controlled except the influence of sunlight.

METHODS EMPLOYED IN THE GROWTH AND PREPARATION OF SAMPLES

Plants Used.—The soybean and the cowpea were selected for this work because of their adaptability to the conditions under which it was necessary to carry on the investigations. The rapidity and habit of growth, as well as the nodule formation of these plants, makes them especially desirable for experiments of this sort. The cowpea is an important crop, especially in southern Illinois, while the soybean is being grown more and more each year in central and northern Illinois. The seed used in all the experiments conducted was produced in 1910 on some of the experimental fields of this station. The soybeans were of the Medium Green variety, the cowpeas, the Whippoorwill.

Methods Used in Growing the Plants.—4.7 kilos of pure nitrogen-free sand, in which 10 grams of chemically pure calcium carbonate had been thoroly mixed, were placed in each of a number of 6-inch cylin-

PLATE XIV.—TYPICAL JAR OF FIVE COWPEAS BEING GROWN FOR SAMPLES

drical glass battery jars. These jars were painted black in order to keep the light from the roots and check the growth of algae. A moisture content of 12 percent was maintained thruout the growth of the plants. This was done by weighing each jar at least every week, sometimes every four days, and adding sufficient nitrogen-free, distilled water[1] to restore the original weight of the jar. During the interim each jar was given the same amount of water.

Mineral plant food in solution was applied once a week. Each jar was given the following solutions:

```
10 cc. each of—
    25 grams CaH₄ (PO₄)₂  per 2500 cc. water
    20 grams MgSO₄        per 2500 cc. water
    50 grams K₂SO₄        per 2500 cc. water
1 cc. of—
    .1 gram  FeCl₃        per 250 cc. water
```

These amounts were diluted with water and added at the same time that the plants were made up to weight. The jars in several series were kept out of doors during the pleasant days, but all plants were kept in the greenhouse during the night.

Planting and Inoculation.—Five seeds of average size were selected and planted in each jar. In the earlier work, in order to insure a proper germination of the five seeds and avoid the possibility of some decaying and leaving organic matter in the sand, moistened filter papers were placed over the seeds until germination was assured, when the seeds were covered with one-half inch of sand. Later this method was found unnecessary owing to the excellent germination of the seeds; and by starting more jars than needed in the series, the required number containing five plants was insured. By always covering the seeds with the same amount of sand, more uniform plants were secured.

Inoculation was attained by the following method: Plants were grown in a soil in which they would form nodules. These nodules were then removed from the plant, thoroly washed, and dipped in alcohol. After burning off the alcohol by passing the nodules thru a flame, they were crushed in sterile distilled water. The inoculum was then diluted to about a liter and 5 cc. applied to each seed just before it was covered with sand. When this method of infection was carried out, no failures were experienced and an abundance of nodules was always obtained.

Uninoculated material was obtained by planting a few jars similarly to the inoculated, except that the seeds were dipped in alcohol and the alcohol then burned off, and that a sterile spatula was used in planting. The sand in these jars was not sterilized, but the jars were placed in vessels of water in order to prevent possible infec-

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[1]Whenever water is mentioned in this publication nitrogen–free distilled water is always meant, unless otherwise stated.

tion by ants and red spiders. This method gave very good results; in no case did inoculation occur.

Harvesting Samples.—As soon as the seed coats were free from the young seedlings, they were removed from the jars, labeled according to the jars from which they came, and later were analyzed with the roots from that jar. In like manner the cotyledons and all the leaves which had dropped were analyzed with the tops from the corresponding jar.

The periods at which the plants were harvested were regulated according to the development of the leaves. Harvests were made from jars as nearly uniform as possible, in most cases selected in triplicate. The jars were taken to the laboratory, where the tops of the plants were cut one inch above the surface of the sand and placed in a suitable receptacle to air-dry. A stream of water was then carefully directed on the jars in order to wash the sand from the roots. After all the visible nodules had been carefully removed with forceps, counted, and placed away to air-dry, the roots were also placed away to air-dry.

Laboratory numbers were given as follows: odd hundreds to soybean series, even hundreds to cowpeas; the tens to the number of the harvest; and the units 1, 2, 3, to tops; 4, 5, 6, to roots; and 7, 8, 9, to nodules. Thus, 129 refers to soybean nodules of the second harvest. The roots of the same are numbered 126, and the tops, 123.

Preparation of the Sample.—After complete air-drying, all the samples were ground so that they would pass thru a 100-mesh sieve. No difficulties were experienced in the grinding except with that part of the stem which extends a little above and below the surface of the sand. At this place where the plant needs the greatest strength to sustain its upright position, the fiber is very tough. The difficulty in grinding this part of the stem was overcome by adding some grains of pure sand before grinding the tops and roots. All possible care was exercised in grinding, yet some slight loss was inevitable. This was especially true with the soybean leaves, as they are pubescent and small hairs will sometimes float in the air. However, it is doubtful whether the error due to this loss was relatively as great as that due to chemical manipulation. As the results given in the tables all refer to milligrams of nitrogen per jar, the presence of sand in some samples does not affect them. After the samples had been ground they were placed in air-tight jars until needed for analysis, when the total weight of each was taken.

ANALYTICAL METHODS

Total Nitrogen.—When determining total nitrogen, duplicate subsamples for each determination on each sample were weighed out at the same time in order to avoid error from possible moisture changes. For the tops and roots .5 gram was taken and for the

nodules .1 or .2 gram. The total nitrogen was determined by the Jodl-bauer method.[1] Glass beads were used to prevent bumping in digest-ing. The hydrochloric acid and the ammonium hydroxid used in titrating were one-tenth normal. Lacmoid was used thruout as the indicator. The roots were not so easily oxidized as the other parts, but the use of permanganate to complete the oxidization was avoided, except in the case of a very few determinations.

Insoluble Nitrogen.—In determining insoluble nitrogen, a .2-gram sample was weighed out and placed in a 400-cc. shaker bottle, 150 cc. of water was added, and the bottle was then placed in a mechanical shaker for three hours. In some cases a smaller sample was used, es-pecially with the nodules, and sometimes with the roots; in these cases a smaller amount of water was used. The contents of the bottle were then filtered onto an S. & S. filter paper and platinum cone, suction being used when necessary. The residue and filter paper were trans-ferred to a Kjeldahl flask and the insoluble nitrogen determined by the Kjeldahl method.

Soluble Nitrogen.—The total soluble nitrogen was obtained by ad-ding P.T.A.[2] and Other nitrogen. In the few cases where distillation was made with sodium hydroxid, the nitrogen obtained is also in-cluded under the soluble nitrogen.

Nitrogen by Distillation with NaOH.—In several of the harvests of the first two series, the filtrate from the insoluble nitrogen was dis-tilled with sodium hydroxid. The nitrogen obtained by this treatment is reported as nitrogen by sodium hydroxid. This nitrogen may rep-resent various compounds, as will be explained later.

P.T.A. Nitrogen.—In order to determine P.T.A. nitrogen, the filtrate from the insoluble residue was made up to 200 cc. with water. Five grams of concentrated sulfuric acid per 100 cc. of solution were added and then 10 cc. of a solution containing 20 grams of P.T.A. and 5 grams of sulfuric acid per 100 cc. of water. In the beginning 30 cc. of P.T.A. solution was used; later this amount was reduced to 10 cc. and in some cases to only 5 cc. with the roots. This precipita-tion was made in the cold and the solution allowed to stand for forty-eight hours in order to obtain a complete precipitation of arginine. At the end of that time the precipitates were filtered off thru S. & S. filter papers and washed with a solution containing 2.5 grams of P.T.A. and 5 grams of sulfuric acid per 100 cc. Later, washing was carried out by using part of the mother liquor. The precipitates on the filters when thoroly washed and dried were transferred to Kjel-dahl flasks, and the nitrogen determined by the Kjeldahl method.

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[1]The Jodlbauer method was used at first, as it was thought nitrates might be present, but later it was discontinued except for determining total nitrogen.

[2]See note to Table 4, page 500.

Other Nitrogen.—In determining Other nitrogen, the filtrate from the P.T.A. precipitate, after having been evaporated to about 30 cc. and then made up to 50 cc., was divided into two parts in the first series, and a Kjeldahl determination made on one half and an amino-nitrogen determination on the other. This proved unsatisfactory because of the small amount of nitrogen present in the filtrate for an amino-nitrogen determination. Owing to the excess of P.T.A. in this filtrate, serious bumping occurred during digestion. Altho glass funnels were placed in the necks of the flasks to prevent loss, some determinations were lost. Digestion was continued for four to seven hours with this filtrate.

A few modifications of the above methods are considered in connection with the series in which they occurred. In all the analytical work the reagents were carefully and constantly checked up for nitrogen, altho the methods were applied under the same conditions at all times.

DISCUSSION OF SOME OF THE METHODS USED

The insoluble nitrogen represents certain proteins and probably other insoluble nitrogenous compounds. The nitrogen obtained by distillation of the filtrate from the above might represent nitrogen from acid amides or volatile organic bases or basic amino nitrogen (arginine and cystine). The presence of volatile organic bases was eliminated by qualitative tests. Thus it would seem that the nitrogen found by the distillation represents an amide or basic nitrogen. The presence of asparagine in soybean seedlings has already been pointed out, but it cannot be assumed that the nitrogen thus determined was asparagine, as arginine and cystine may have been present.

P.T.A., as already stated, precipitates various nitrogenous compounds. The reagent, however, does not completely precipitate alkaloids, peptones, proteoses, peptides, and diamino acids (basic nitrogen); further, Van Slyke[1] has shown that the diamino acids are appreciably soluble in this reagent. Osborne and Harris[2] have demonstrated that P.T.A., while subject to various criticisms, nevertheless, when used under constant conditions, gives very good comparative results.

The filtrate from the P.T.A. precipitate contains all soluble nitrogen not precipitated by P.T.A. This nitrogen may be made up of amino acids, pigment nitrogenous compounds, and possibly other nitrogenous compounds.

QUALITATIVE TESTS

A large number of plants and parts thereof, grown under the same conditions as those harvested for quantitative determinations

[1]Van Slyke: Jour. Biol. Chem. (1911-12), 10, 15-56.
[2]Osborne and Harris: Jour. Am. Chem. Soc. (1903), 25, 323-333.

were tested for ammonia, nitrites, and nitrates. Nessler's reagent was used to test for ammonia and diphenylamine sulfuric acid for nitrites and nitrates. *All these tests were negative.* Ten grams of tops were treated with 800 cc. of water in a liter flask. The solution was boiled but no ammonia was obtained. Upon the treatment of the filtrate from this solution with sodium hydroxid, a large amount of ammonia was found but no volatile organic bases, as the distillate gave the typical test for ammonium chlorid when absorbed in hydrochloric acid and left no carbonaceous residue. Zinc sulfate gave no precipitate in this filtrate. The filtrates from the P.T.A. precipitates were tested in the Van Slyke apparatus with results which indicate that some of the nitrogen in the filtrate was in the form of primary amines.

Since the completion of these experiments, a study of the total amino nitrogen in the seedlings of the Alaska pea, as obtained by the Van Slyke apparatus, has been reported by Thompson.[1] His results indicate that as high as 43.3 percent of the total nitrogen may be in the form of primary amines. The percentage of amino nitrogen found in the seed was only .088, increasing in the seven-day seedlings to 28.27.

SERIES 100 (SOYBEANS)

The conditions under which the soybeans used in Series 100 were grown have already been explained (see page 523). Their development at each of the five harvests is shown in Table 14.

TABLE 14.—DEVELOPMENT OF SOYBEANS: SERIES 100

Planted	Harvested	Age, days	Leaves and pods per plant	Height, inches	Nodules per 15 plants
Mar. 18, 1911	Apr. 25	38	4 leaves	6	265
,, ,, ,,	May 10	53	6 ,,	11	1 061
,, ,, ,,	May 17	60	8 ,,	14	987
,, ,, ,,	May 24	67	10–12 leaves; an average of 5.4 pods	16–17	1 154
,, ,, ,,	May 31	74	10–12 leaves; beans formed in pods	16–17	1 354

The total nitrogen determinations in the various parts of the plants of this series at different stages of growth, together with the nitrogen fixed at each of these stages, are given in Table 15. The amount of the nitrogen fixed was determined by subtracting, from the

[1]Thompson: Jour. Am. Chem. Soc. (1915), **37**, 230–235.

total nitrogen found, the average nitrogen content of five soybean seeds as shown by the individual analyses of twenty seeds. Nearly all the figures in this table represent the average of six determinations.

TABLE 15.—TOTAL NITROGEN IN VARIOUS PARTS OF SOYBEANS AND FIXATION AT DIFFERENT PERIODS: SERIES 100

(Milligrams per jar of five plants)

Harvest	Lab. Nos.	Nitrogen in tops	Nitrogen in roots	Nitrogen in nodules	Nitrogen in whole plants	Nitrogen in seeds	Nitrogen fixed
1	111–112 114–115 117–118	87.10	13.35	28.04	128.49	57.30[1]	71.19
2	121–129	204.59	22.70	47.10	274.39	57.30	217.09
3	131–139	286.91	43.44	82.95	413.30	57.30	356.00
4	141–149	356.52	40.15	60.40	457.07	57.30	399.77
5	151–159	247.82	30.82	54.56	333.20	57.30	275.90

[1]It would be preferable to use the analyses of uninoculated plants as a check rather than the analyses of seeds.

These figures need very little explanation. The results of the first four harvests show a gradual increase in the amount of nitrogen fixed. The low results obtained at the last harvest are in accord with the results of Wilfarth and Wimmer[1] and Penny and MacDonald.[2]

The separation of the nitrogen compounds into the various groups was carried out in this series as follows: The whole sample was dried for four hours at 50°C. both before and after grinding. The subsamples were weighed out and placed in 250-cc. beakers; 100 cc. of water was then added and the whole heated to boiling and filtered while hot. Suction was used in filtration, and the washing was done with 50 cc. of hot water. The residue was Kjeldahlized as usual. The filtrates from some of the harvests were treated with 10 cc. of sodium hydroxid and distilled. The residual liquid in the Kjeldahl flask was transferred to a 350-cc. beaker and the excess alkali neutralized with sulfuric acid, after which the regular P.T.A. method was applied. The precipitates obtained by P.T.A. were characteristic in their behavior. After the reagent had been added some one or two hours, a voluminous grayish white precipitate appeared, sometimes colored a yellowish green, and very gradually settled to the bottom in a very thin layer, leaving the supernatant liquid yellowish green in the case of

[1]Wilfarth and Wimmer: Landw. Vers. Stat. (1906), **63**, 1–70.
[2]Penny and MacDonald: Del. Agr. Exp. Sta. Bul. **86**, 35.

the tops, slightly straw colored to colorless in the case of the roots, and colorless in the case of the nodules.

The amount of the precipitate from the determination with the tops was much greater than that with the roots and nodules. Caution was exercised in all the determinations not to allow losses or changes due to bacterial action. A few drops of chloroform were placed on the filter and in the filtrate when the determinations were sufficiently long to be liable to bacterial action.

The results of the separations are shown in Table 16. Here again, as in the case of the total nitrogen determinations, most of the figures represent the average of two determinations made upon samples from each of three jars. The total soluble nitrogen was obtained by the addition of the various determined soluble forms. The total nitrogen reported in the last column is the sum of all the separations made. These results will be discussed later with those of the other series.

TABLE 16.—NITROGEN SEPARATIONS: SERIES 100 (SOYBEANS)

(Milligrams per jar of five plants)

Har- vest	Lab. Nos.	Part	Insol- uble nitro- gen	Total solu- ble nitro- gen	NaOH nitro- gen	P.T.A. nitro- gen	Other nitro- gen	Total nitro- gen
1	111–112	Tops	61.52	24.39	....	4.16	20.23	85.91
	114–115	Roots	8.90	5.00	....	.85	4.15	13.90
	117–118	Nodules	15.72	11.61	....	3.54	8.07	27.33
2	121–123	Tops	135.15	37.99	....	8.11	29.88	173.14
	124–126	Roots	15.49	5.67	....	.48	5.19	21.16
	127–129	Nodules	32.83	16.03	....	9.66	6.37	48.86
3	131–133	Tops	146.79	140.12	....	25.63	114.49	286.91[1]
	134–136	Roots	27.03	16.42	....	.93	15.49	43.45
	137–139	Nodules	47.95	35.00	....	18.55	16.45[2]	82.95[1]
4	141–143	Tops	183.35	134.26	17.86	25.96	90.44	317.61
	144–146	Roots	26.14	12.93	2.49	.85	9.59	39.07
	147–149	Nodules	31.77	27.27	2.38	15.35	9.54	59.04
5	151–153	Tops	151.68	95.32	12.02	29.31	53.99[2]	247.00[1]
	154–156	Roots	21.55	14.38	1.34	1.38	11.66	35.93
	157–159	Nodules	29.23	27.21	2.00	12.13	13.08	56.44

[1]Taken from Table 15.
[2]Obtained by difference.

The accompanying graph (Plate XV) shows clearly the relationship in which the soluble and the insoluble nitrogen exist at the various periods of growth. In this series the first harvest was not made for thirty-eight days, and therefore the period at which fixation began is not shown. Attention has already been called to the low nitrogen fixa-

SOLUBLE AND INSOLUBLE NITROGEN
IN TOPS ROOTS AND NODULES OF SOYBEANS
AT
DIFFERENT PERIODS OF DEVELOPMENT

Series 100
LEGEND
SOLUBLE ☐ INSOLUBLE ■

TOPS

ROOTS
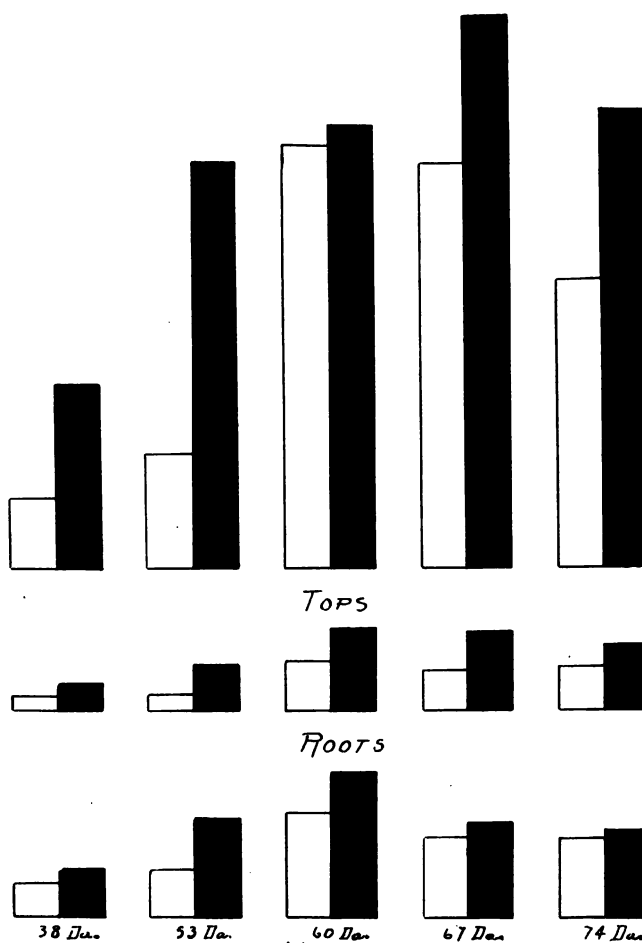
38 Da.   53 Da.   60 Da.   67 Da.   74 Da.
NODULES

PLATE XV

tion found at the last harvest in this series. More results are necessary to confirm the supposition of a possible loss in the total nitrogen.

The importance of the amount of soluble nitrogenous compounds at the various stages of growth has not yet been emphasized. Preliminary studies have shown this soluble nitrogen to be much more rapidly converted into ammonia and nitrates than the insoluble nitrogen. This would seem to have a direct bearing upon practical methods of handling leguminous crops in rotations when the shortest time must intervene between the turning under of the legume and the planting of the next crop. It would seem desirable to choose that period when the greatest amount of soluble nitrogen exists.

SERIES 500 (SOYBEANS)

Soybeans were used in Series 500. The plants were placed out of doors during pleasant days in August and September. From Table 17, showing the development at the four harvests, it will be seen that these plants made a more rapid growth than those in Series 100.

TABLE 17.—PLANT DEVELOPMENT: SERIES 500 (SOYBEANS)

Planted	Harvested	Age, days	Leaves and pods per plant	Height, inches	Nodules per 15 plants
Aug. 8, 1911	Aug. 22	14	3 leaves	7	30[1]
,, ,, ,,	Aug. 30	22	4 ,,	10	278
,, ,, ,,	Sept. 8	30	6 ,,	14	370
,, ,, ,,	Sept. 19	41	7-8 ,, ; 6 one-inch pods	14	247 (large)

[1]For ten plants.

The total nitrogen determinations for this series are shown in Table 18. These results agree with those shown in Table 15, altho they represent earlier stages of development.

TABLE 18.—TOTAL NITROGEN IN VARIOUS PARTS OF SOYBEANS AND FIXATION AT DIFFERENT PERIODS: SERIES 500

(Milligrams per jar of five plants)

Harvest	Lab. Nos.	Nitrogen in tops	Nitrogen in roots	Nitrogen in nodules	Nitrogen in whole plants	Nitrogen in seeds	Nitrogen fixed
1	511–519	46.99	8.50	.35	55.84	57.30[1]	(−1.46)
2	521–529	47.32	11.08	11.05	69.45	57.30	12.15
3	531–539	96.96	9.76	17.81	124.53	57.30	67.23
4	541–549	205.40	18.65	26.98	251.03	57.30	193.73

[1]This figure is approximate rather than exact. See note to Table 15, page 529.

The separations in this series differed from those in Series 100 in that in this case a cold-water extract was made. The sub-samples

were placed in shaker bottles and the same amount of water added as in the former series; the bottles were then put in a mechanical shaker for three hours. This method was considered to be more in accord with natural conditions than the one used in the former series. The nodules were filtered thru a diatomaceous earth filter.

The figures in Table 19 were obtained in the same manner as those in Table 16.

TABLE 19.—NITROGEN SEPARATIONS: SERIES 500 (SOYBEANS)

(Milligrams per jar of five plants)

Har-vest	Lab. Nos.	Part	Insol-uble nitro-gen	Total soluble nitro-gen	NaOH nitro-gen	P.T.A. nitro-gen	Other nitro-gen	Total nitro-gen
1	511–512	Tops	19.48	25.90	3.16	3.31	19.43	45.38
	514–515	Roots	4.42	4.48	.95	.33	3.20	8.90
	517–518	Nodules	....	....	...	...	....	....
2	521–523	Tops	29.60	17.34	1.18	4.96	11.20	46.94
	524–526	Roots	7.97	2.14	.00	.47	1.67	10.11
	527–529	Nodules	....	....	...	...	....	....
3	531–533	Tops	70.04	31.64	2.67	6.48	22.49	101.68
	534–536	Roots	8.56	2.29	.00	.70	1.59	10.85
	537–539	Nodules	16.75	1.29	.00	.33	.96	18.04
4	541–543	Tops	115.39	76.11	...	10.68	65.43	191.50
	544–546	Roots	13.16	7.35	...	2.06	5.29	20.51
	547–549	Nodules	22.55	2.46	...	.26	2.20	25.01

SERIES 700 (SOYBEANS)

Soybean seeds were planted on September 6 for this series, but owing to their damping off, the jars were replanted on September 13. The seedlings that damped off were tested for ammonia, nitrites, and nitrates, with negative results. The conditions of the plants at the various harvests are shown in Table 20.

TABLE 20.—PLANT DEVELOPMENT: SERIES 700 (SOYBEANS)

Planted	Harvested	Age, days	Leaves per plant	Height, inches	Nodules per 15 plants
Sept. 13, 1911	Sept. 25	12	2 leaves	5	Nodules present too small to remove
,, ,, ,,	Oct. 6	23	3 leaves partially developed	8–9	254
,, ,, ,,	Oct. 14	31	5 leaves	10–12	229
,, ,, ,,	Oct. 25	42	7 leaves	12	288

The figures reported in Table 21 present the average of duplicates of composite samples which included the whole of the material from three jars. As will be seen, these results are concordant with those of the two series already considered.

TABLE 21.—TOTAL NITROGEN IN VARIOUS PARTS OF SOYBEANS AND FIXATION AT DIFFERENT PERIODS: SERIES 700

(Milligrams per jar of five plants)

Har-vest	Lab. Nos.	Nitro-gen in tops	Nitro-gen in roots	Nitro-gen in nodules	Nitro-gen in whole plants	Nitro-gen in seeds	Nitro-gen fixed
1	711–719	40.08	8.88	....	48.96	57.30[1]	(−8.34)
2	721–729	48.00	8.93	7.21	64.14	57.30	6.84
3	721–739	68.32	11.38	8.97	88.67	57.30	31.37
4	741–749	110.70	20.55	17.22	148.47	57.30	91.17

[1]See note to Table 15, page 529.

The figures in Table 22 showing the nitrogen separations were obtained in the same manner as those reported for the nitrogen fixation in Table 21; that is to say, they are the averages of duplicates of composite samples.

TABLE 22.—NITROGEN SEPARATIONS: SERIES 700 (SOYBEANS)

(Milligrams per jar of five plants)

Har-vest	Lab. Nos.	Parts	Insol-uble nitro-gen	Solu-ble nitro-gen	P.T.A. nitro-gen	Other nitro-gen	Total nitro-gen
1	711	Tops	9.94	28.03	4.70	23.33	37.97
	714	Roots	3.87	4.95	.39	4.56	8.82
	717	Nodules	....	....	....	....	....
2	721	Tops	23.64	21.56	11.96	9.60	45.20
	724	Roots	5.50	2.55	.77	1.78	8.05
	727	Nodules	....	....	....	....	....
3	731	Tops	26.50	29.05	13.75	15.30	55.55
	734	Roots	7.30	3.76	1.10	2.66	11.06
	737	Nodules	6.30	1.79	.10	1.69	8.09
4	741	Tops	46.45	61.97	23.75	38.22	108.42
	744	Roots	10.67	6.31	.61	5.70	16.98
	747	Nodules	14.52	2.70	.00	2.70	17.22

The close agreement of the results of Series 700 and 500 is very evident in the data presented. By reference to the accompanying graph (Plate XVI) it will be seen more easily than in the tabular form that the soluble nitrogen predominates in the early growth of the seedling. The amount decreases during this period, however, while

Soluble and Insoluble Nitrogen
in Tops, Roots and Nodules of Soybeans
at
Different Periods of Development
Soluble ☐ Insoluble ■

Series 700

Tops

Roots

12 Da.   23 Da.   31 Da.   42 Da.

Nodules

Series 500

Tops

Roots

14 Da.   22 Da.   30 Da.   41 Da.

Nodules

Plate XVI
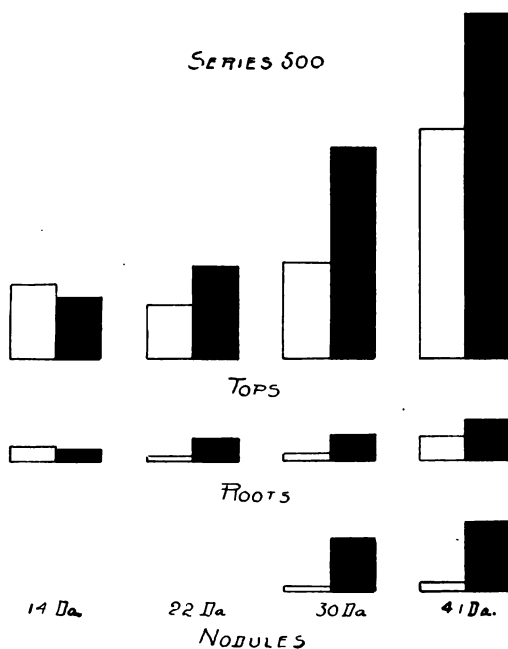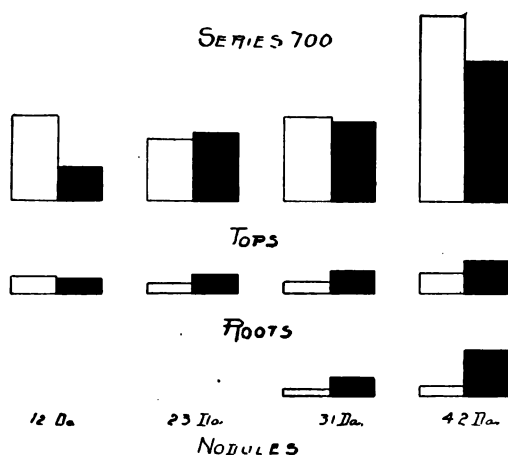
the insoluble nitrogen always increases. This is true of the roots as well as the tops. It is during the period between the twelfth and the twenty-second days that nitrogen fixation begins, according to measurements by the most accurate chemical methods. Detailed studies are now being made of the exact time when fixation begins.

SERIES 600 (COWPEAS)

Cowpeas were used for Series 600. Owing to the smaller nitrogen content of cowpea seeds, the plants show a need of nitrogen much sooner than soybeans, and are therefore perhaps better suited to experimentation of this sort. The plants grown in this series are comparable with the soybeans in Series 500 as regards time and conditions of growth. The data in Table 23 show the development of the cowpeas when harvested.

TABLE 23.—PLANT DEVELOPMENT: SERIES 600 (COWPEAS)

Planted	Harvested	Age, days	Leaves per plant	Height, inches	Nodules per 15 plants
Aug. 8, 1911	Aug. 22	14	3 leaves	7	450 (very small)
,, ,, ,,	Aug. 30	22	4 ,,	10	892 (small)
,, ,, ,,	Sept. 8	30	5 ,,	12	1074
,, ,, ,,	Sept. 19	41	6-7 ,,	13-14	2062
,, ,, ,,	Oct. 5	58	8 ,,	14	1992

The results given in Table 24 show a fixation of nitrogen at the end of fourteen days from the time the seeds were placed in the sand. The increased fixation is greater with the cowpeas in this series than with the soybeans in the corresponding series (500). The other general tendencies appear to be the same as in the other series.

TABLE 24.—TOTAL NITROGEN IN VARIOUS PARTS OF COWPEAS AND FIXATION AT DIFFERENT PERIODS: SERIES 600

(Milligrams per jar of five plants)

Harvest	Lab. Nos.	Nitrogen in tops	Nitrogen in roots	Nitrogen in nodules	Nitrogen in whole plants	Nitrogen in uninoculated plants	Nitrogen fixed
1	611-619	29.11	9.05	1.96	40.12	36.74[1]	3.38
2	621-629	45.46	10.25	9.22	64.93	36.74	28.19
3	631-639	91.63	16.64	18.52	126.79	36.74	90.05
4	641-649	188.40	30.28	42.33	261.01	36.74	224.27
5	651-659	439.64	73.73	64.25	577.62	36.74	540.88

[1]This figure was used as it is a little larger than the average nitrogen content for the seeds, 34.70 milligrams, which would make even a greater fixation appear at the harvest.

The results of the separations are shown in Table 25. The figures were obtained in the same manner as those in Series 700.

TABLE 25.—NITROGEN SEPARATIONS: SERIES 600 (COWPEAS)

(Milligrams per jar of five plants)

Harvest	Lab. Nos.	Part	Insoluble nitrogen	Total soluble nitrogen	P.T.A. nitrogen	Other nitrogen	Total nitrogen
1	611-613	Tops	15.83	11.62	3.68	7.94	27.45
	614-616	Roots	6.92	2.13	.74	1.39	9.05
	617-619	Nodules	....	....	....	....	....
2	621-623	Tops	23.73	21.76	5.88	15.88	45.49
	624-626	Roots	9.18	1.89	.42	1.47	11.07
	627-629	Nodules	7.54	2.26	.92	1.34	9.80
3	631-633	Tops	58.67	32.54	10.55	21.99	91.21
	634-636	Roots	11.18	5.32	1.90	3.42	16.50
	637-639	Nodules	13.69	4.83	1.22	3.61	18.52
4	641-643	Tops	97.82	93.47	32.49	60.98	191.29
	644-646	Roots	21.54	7.61	2.51	5.10	29.15
	647-649	Nodules	26.74	15.59	9.08	6.51	42.33
5	651-653	Tops	216.90	226.50	58.50	168.00	443.40
	654-656	Roots	52.35	19.87	5.17	14.70	72.22
	657-659	Nodules	32.36	31.89	20.10	11.79	64.25

The results of this series are also presented in a graph (Plate XVII). The curve of the soluble nitrogen does not show a decrease, possibly because the change upward had taken place before the end of the first fourteen days, when the first data were taken, as cowpeas show an early fixation of nitrogen and develop extremely rapidly under normal conditions. The same general tendencies hold thruout this series as in the others in respect to the increase of the soluble and insoluble nitrogen. When the soluble and the insoluble nitrogen ratios are considered, the results in general agree very closely in all the series regardless of time of growth and kind of legume.

DISCUSSION OF TABLES

The results of the total nitrogen determinations of the four series show that, as an average of eighteen harvests, 74 percent of the nitrogen of the cowpeas and soybeans was in the tops, the remaining 26 percent being divided between the roots and the nodules. The figures show that in the first periods most of the 26 percent was in the roots, while later the nodules in some cases contained 18 of the 26 percent. In nine out of seventeen harvests, the nodules contained more nitrogen than the roots of the same plants.

The data showing the average daily fixation of nitrogen for five plants in the various series during the different growing periods are presented in Table 26.

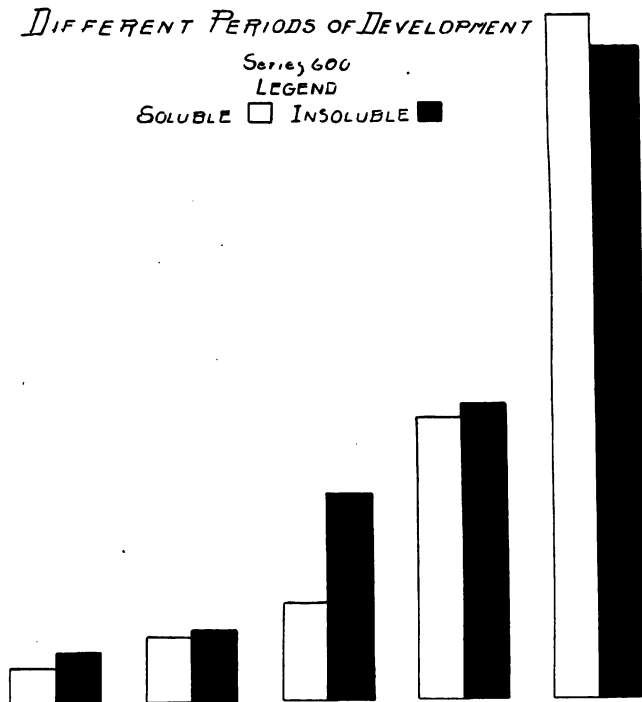SOLUBLE AND INSOLUBLE NITROGEN
IN TOPS, ROOTS AND NODULES OF COWPEAS
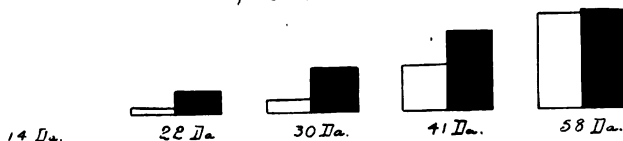AT
DIFFERENT PERIODS OF DEVELOPMENT

Series 600
LEGEND
SOLUBLE ☐ INSOLUBLE ■

TOPS

ROOTS

14 Da.   22 Da.   30 Da.   41 Da.   58 Da.

NODULES

PLATE XVII

Table 26.—Average Daily Fixation of Nitrogen in All Series

Series	Periods in days	Milligrams per jar of 5 plants
100 (Soybeans)	0–38	1.87
	38–53	9.72
	53–60	19.84
	60–67	6.16
	67–74	(−17.69)
500 (Soybeans)	0–14	.00
	14–22	1.51
	22–30	6.88
	30–41	11.50
700 (Soybeans)	0–12	.00
	12–23	.62
	23–31	3.06
	31–42	5.43
600 (Cowpeas)	0–14	.24
	14–22	3.10
	22–30	7.73
	30–41	14.02
	41–58	17.44

The results included in Table 26 seem to indicate that the period during which the greatest total accumulation of atmospheric nitrogen takes place occurs between the fortieth and sixtieth days and coincides closely with the period just previous to seed formation. The greatest rate of increase of fixation and assimilation in these series occurred in the early periods of growth. A comparison of Series 100 with the other series indicates that the growth of the plant is closely related to the rate and the amount of nitrogen fixed. The plants of Series 100 grew much slower than the others. Those of Series 500 and 600 made the greatest growth in a given period, having had the advantage of the most favorable growing season, more especially for the cowpea, which requires a higher temperature than the soybean for optimum growth.

The fixation in Series 600 for the whole period of fifty-eight days was 540.88 milligrams per five plants, or an average daily fixation of 9.32 milligrams. The greatest fixation during any one period, as is evident from Table 26, occurred with the soybeans in Series 100 between the fifty-third and sixtieth days, when the daily average was 19.84 milligrams per five plants, or nearly 4 milligrams per plant per day. If this figure is calculated to an acre basis, allowing a stand of four beans per square foot, an accumulation equivalent to one and a half pounds of nitrogen per acre per day is shown.

The average percentages of soluble nitrogen in the four series in terms of total nitrogen in the particular part of the plant, may be of some interest, altho it will be seen from the accompanying graphs

that the amount depends upon the stage of growth when the harvest is made. These percentages were as follows:

TABLE 27.—PERCENTAGES OF SOLUBLE NITROGEN IN EACH SERIES
AS AN AVERAGE OF ALL HARVESTS

(On the basis of total nitrogen in the given part)

Series	Tops	Roots	Nodules
100 (Soybeans)	35.9	34.7	42.3[1]
700 (Soybeans)	57.7	39.5	18.9
500 (Soybeans)	41.2	32.4	8.5
600 (Cowpeas)	45.0	27.5	34.0

[1]The nodules in this series were not filtered thru a diatomaceous earth filter but thru an ordinary filter and are therefore not included in the average given in the conclusions.

The figures for Series 100 represent soluble nitrogen obtained in a hot-water extract. Series 500 and 600 are comparable with the exception of one being soybeans and the other cowpeas. The great difference in the solubility of the nitrogen in the nodules is particularly noticeable.

There was a gradual increase in the soluble nitrogen in the nodules of Series 600 from the first harvest to the last, the percentages on the basis of total nitrogen being 23, 26, 37, and 49. A fact not brought out in the figures showing the soluble nitrogen is that in Series 700 and 500 an extremely high soluble-nitrogen content was found in the tops and the roots at the first harvest. In Series 700 the percentage in the tops was 74, in Series 500, 57; while in the roots in Series 700 the percentage was 56, and in Series 500, 50.

On the basis of total nitrogen, the percentage of Other nitrogen in each series, as an average of all harvests, was as shown in Table 28. Other nitrogen is the difference between the total soluble nitrogen and that precipitated by P.T.A. and NaOH. It has been shown that a part of this nitrogen consists of amino acids, but as yet the total amount is unknown.

TABLE 28.—PERCENTAGES OF OTHER NITROGEN IN EACH SERIES
AS AN AVERAGE OF ALL HARVESTS

(On the basis of total nitrogen in the given part)

Series	Tops	Roots	Nodules
100 (Soybeans)	25.1	29.3	...
700 (Soybeans)	36.1	32.8	18.2
500 (Soybeans)	30.7	23.1	6.9
600 (Cowpeas)	28.3	17.2	16.6

The percentages of nitrogen precipitated by P.T.A. were as shown in Table 29. The variations as regards the tops are not easily explainable. Undoubtedly there is a larger error in the P.T.A. determinations than in the others. The nodules of Series 600 contained

large amounts of nitrogen which were precipitated by this reagent, the percentages at the harvests, from the second to the last, on the basis of total nitrogen being 9, 7, 21, and 31.

TABLE 29.—PERCENTAGES OF P.T.A. NITROGEN IN EACH SERIES
AS AN AVERAGE OF ALL HARVESTS

(On the basis of total nitrogen in the given part)

Series	Tops	Roots	Nodules
100 (Soybeans)	7.00	3.0	...
700 (Soybeans)	21.10	6.6	1.0
500 (Soybeans)	6.75	6.0	1.4
600 (Cowpeas)	13.40	7.3	17.0

The nitrogen obtained by distillation with sodium hydroxid apparently is not precipitated by P.T.A., as the percentage of Other nitrogen decreases without exception when sodium hydroxid is used. However, no definite conclusions can be drawn regarding the use of sodium hydroxid.

CONCLUSIONS

PART I

1.   The experiments reported show conclusively that the cowpea and the soybean utilize atmospheric nitrogen thru their roots and not thru their leaves.   No combined nitrogen could have been assimilated in these gas experiments.

PART II

2.   The total nitrogen determinations show that about 74 percent of the nitrogen of cowpeas and soybeans at the time of harvest is in the tops, while the remainder is distributed between the roots and the nodules.   In the earlier periods the roots contain the larger part, while later they contain much the smaller part.

3.   The percentage of soluble nitrogen in soybeans and cowpeas varies with the different parts of the plant and with the period of growth.   In these experiments the soluble nitrogen, as an average, constituted in the tops about 45 percent of the total nitrogen; in the roots, 34 percent; in the nodules of the soybeans, 14 percent, and in the nodules of the cowpeas, 34 percent.

4.   Phosphotungstic acid usually precipitates some form of nitrogen.   In some cases the amounts precipitated vary widely, while in others the agreement is close.   In these series the nitrogen precipitated by phosphotungstic acid averaged in the tops of both soybeans and cowpeas about 12 percent of the total nitrogen; in the roots, 5.5 percent; in the nodules of the soybeans 1 percent, and in the nodules of the cowpeas, 17 percent.

5. Other forms of soluble nitrogen than those precipitated by phosphotungstic acid and sodium hydroxid occur. In these series they constituted as an average in the tops of both soybeans and cowpeas about 68 percent of the soluble nitrogen; in the roots, 77 percent; in the nodules of soybeans, 89 percent, and in the nodules of cowpeas, 53 percent.

6. Fixation takes place at a very early period in the growth of the seedling—sometimes within fourteen days. It is rapid in some cases, especially with cowpeas.

7. Plants grown under the conditions of these experiments contain no ammonia, nitrites, or nitrates, as measured by the most accurate chemical methods.

It is fully recognized that this work is incomplete, yet it is hoped that the study may aid in stimulating interest in some of these fundamental problems. The lack of development of the various chemical methods used is partially responsible for some of the difficulties experienced in their application. The survey presented of the chemical literature indicates a scarcity of existing knowledge regarding the more fundamental problems concerning nitrogen fixation.

The biological resumé points clearly to the need of more extended research along these lines. This is strikingly noticeable in the bacteriological studies which have been undertaken with *B. radicicola*. Few cases are on record in which the authors actually proved out their cultures at the termination of their investigations by inoculation of a legume of the kind from which the organism originally came.

BIOGRAPHICAL SKETCH

Albert Lemuel Whiting was born in Stoughton, Massachusetts, May 12, 1885. He secured his common-school education in the public schools of that town and then spent one year in mechanical work before entering the Massachusetts Agricultural College in the fall of 1904. From this institution he received the degree of bachelor of science in June, 1908. He immediately accepted a position as assistant agronomist at the Rhode Island Agricultural Experiment Station, where he also instructed in veterinary science in the College. At the same time he served as secretary of the Rhode Island Experimental Union. While there he also took graduate student work in agronomy and botany and in June, 1910, received the degree of master of science. In the fall of 1910 he was granted a fellowship in agronomy in the University of Illinois, which he held during 1910-12. He is now associate in soil biology in that institution.

Dr. Whiting is a member of the following societies: Q.T.V.; Alpha Chi Sigma; the Illinois chapter of Sigma Xi; Society of American Bacteriologists; the American Society of Agronomy; the American Chemical Society; and the American Society for the Promotion of Science.